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(57) Abstract

The present invention provides polypeptides derived from urinastatin having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. The invention also provides polynucleotide sequences encoding these polypeptides, as well as vectors containing these polynucleotide sequences and host cells transformed in these vectors. Also provided is a method of diagnosing and treating a pregnant female individual at risk for impending preterm delivery. The method comprises administering to the female a polypeptide derived from urinastatin having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity, or a polypeptide derived from urinastatin having calcium channel blocking activity and having plasmin/cytokine/trypsin inhibiting activity and lacking elastase inhibiting activity. Also provided is a pharmaceutical composition of these polypeptides and an antibiotic.

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POLYPEPTIDES DERIVED FROM URINASTATIN HAVING CALCIUM CHANNEL BLOCKING ACTIVITY AND THEIR USE TO DELAY PREMATURE DELIVERY

FIELD OF USE

This invention relates to polypeptides derived from urinastatin having calcium channel blocking activity. It also relates to methods of treating females at risk for preterm delivery with these polypeptides.

BACKGROUND

Urinastatin (UTI) is a heat and acid-stable glycoprotein with an approximate molecular weight of 67kD in purified form. UTI is purified from human urine.

Previous functional studies have shown that UTI acts as a protease inhibitor. Urinastatin inhibits various proteases such as trypsin, elastase, granulocyte elastase, chymotrypsin, plasmin, hyaluronidase, amylase and creatine phosphokinase. Some functional domains of the protease inhibitors in UTI have been delineated. The elastase inhibitor region is designated as domain 1B, and the trypsin inhibitor region is designated as 2B (Fig. 1). Urinastatin has also been described as inhibiting cytokine action, stabilizing lysosomal surfaces and suppressing IL-1β-induced reduction of proteoglycan synthesis and superoxide generation.

Because urinastatin inhibits many chemical mediators in inflammation, urinastatin has been evaluated as an anti-inflammatory drug. As such, urinastatin has been proposed for use in a variety of

conditions, such as pancreatitis, septic shock, operative stress, arthritis, thrombosis and preterm delivery. It also has been proposed for use in disseminated intravascular coagulation. Inaba et al., Folia Pharmacol. Japan. 88: 239, 1986.

It has been found that fetal urine and amniotic fluid contain large amounts of UTI (Terao and Kanayama, Sanfuginka Jissai 37: 158, 1988). UTI is also localized in the myometrial cells during pregnancy.

In spite of the advances in health care and particularly perinatology, the preterm delivery of babies continues to be a major public health problem because of its association with infant morbidity and mortality. For example, the results of a multicenter trial spanning several years showed that infants born prematurely, i.e., between 20 and 36 weeks gestation, accounted for 9.6% of births (Copper et al. Amer. J. Obstet. Gynecol. 168: 78, 1993). In that study, 83% of infant deaths occurred in gestations delivering prior to 37 weeks, and 66% involved gestations of less than 29 weeks.

Serious neonatal complications also decrease as the period of gestation increases. The incidence of neonatal respiratory distress syndrome decreases markedly after 36 weeks of gestation. Likewise, the incidence of neonatal patent ductus arteriosus and necrotizing enterocolitis decreases markedly after 32 weeks of gestation. According to Creasy, "high grade intraventricular hemorrhage diminishes rapidly after 27 weeks and is virtually absent after 32 weeks" (Creasy, Amer. J. Obstet. Gynecol. 168: 1223, 1993). Thus, extending the length of pregnancy beyond 32 weeks and preferably beyond 36 weeks could reduce the incidence of neonatal morbidity and virtually eliminate major causes of neonatal mortality.

According to Creasy, the incidence of preterm delivery in the United States is rising. When preterm delivery is defined as births occurring before

37 weeks of gestation, the incidence has risen from 9.4% in 1981 to 10.7% in 1989, accounting for approximately 425,000 of the 4,000,000 annual births in the United States.

There are several problems related to the rising incidence of preterm delivery in the United States. One problem is that physicians are unable to accurately predict which pregnancies are at risk. Unfortunately, the majority of preterm births cannot be related to obvious causes, and even known causes may not necessarily be detectable or correctable. In fact, approximately one half of all preterm births occur in women who are pregnant for the first time and have no known risk factors for preterm delivery.

Even when women complain of symptoms frequently associated with acute risk of preterm delivery, it is often difficult to distinguish harmless symptoms from those associated with imminent prematurity. Many symptoms such as uterine contractions, change in vaginal discharge, abdominal discomfort, pelvic heaviness or change in cervical dimensions (effacement and dilatation) may harmlessly occur as normal variants in some pregnancies, while similar symptoms in other pregnancies can be associated with impending preterm delivery.

The majority of pregnant women who seek unscheduled emergency obstetrical care have complaints of excessive or painful uterine contractions of the uterus. Another frequent complaint is a tightening or pressure sensation which can indicate Braxton Hicks contractions of the uterus. Thus, physicians are often faced with the diagnostic dilemma of differentiating "true" from "false" labor with clinical information of limited diagnostic value (Pircon et al., Amer. J. Obstet. Gynecol. 161:775, 1989). Given the poor predictive power of these clinical signs and symptoms, "clinicians do not have a good discriminator of false versus true labor", resulting in as many as 50% of patients with "false"

labor delivering early (Morrison et al., Amer. J. Obstet. Gynecol. 168:538, 1993).

The presence of fetal fibronectin in cervical or vaginal secretions has been shown to be a predictor for preterm delivery in women with symptoms suggestive of threatened preterm delivery (Lockwood et al., N. Engl. J. Med. 325: 669, 1991). A control group of women with uncomplicated pregnancies who delivered at term rarely had cervicovaginal concentrations of fetal fibronectin greater than 50 ng/ml at weeks 21-37 of gestation. In contrast, approximately 94% of women with preterm rupture of amniotic membranes had significantly elevated fetal fibronectin concentrations. More importantly, about 50% of women with intact amniotic membranes and preterm uterine contractions had elevated concentrations of fetal fibronectin and more than 80% of these women delivered prematurely. Conversely, greater than 80% of women with intact membranes who did not have detectable cervicovaginal fetal fibronectin delivered at term. Thus, fetal fibronectin was demonstrated to be both a sensitive and specific predictor of preterm delivery.

Not surprisingly, the lack of available specific and sensitive indicators of preterm delivery risk among symptomatic women limits the ability of physicians to appropriately treat women judged to be at risk. In addition, there is considerable controversy in the obstetrical community regarding the therapeutic efficacy of available treatment regimens. Of course, assessment of any treatment regimen is complicated by the fact that perhaps as many as half of women diagnosed with preterm labor may not have the disease.

Effective and judicious treatment of preterm labor, especially in earlier gestation, is critical to the development of the fetus. As discussed above, births after 32-34 weeks of gestation are associated with lower rates of neonatal mortality and severe

neonatal morbidity. Thus, prolongation of pregnancy and subsequent reduction of preterm delivery rates might be expected to lower neonatal morbidity and mortality. Unfortunately, in spite of the fact that obstetricians have identified more women as candidates for preterm labor treatment (known as tocolysis) over the last decade, the incidence of preterm delivery has actually increased over the same time span (Creasy, Amer. J. Obstet. Gynecol. 168:1223, 1993). The fact that the preterm delivery rate has not improved over the past decade is due not only to the inability of physicians to accurately identify patients truly in need of treatment but also to the failure of commonly available tocolytic drugs to impede the progress of labor.

Numerous controlled, clinical trials have been conducted to evaluate the clinical merits of various treatment regimens including bedrest, hydration, antibiotics, beta-adrenergic agonists, prostaglandin inhibitors, and calcium antagonists. The cumulative experience of these trials has clearly shown that common strategies for tocolytic intervention do not reproducibly prevent preterm delivery although they may be modestly effective for prolongation of pregnancy.

The role of infection in preterm labor and use of antibiotics has been studied extensively, particularly in the context of premature rupture of the membranes (PROM). For example, a controlled clinical trial of antibiotic treatment with the broad spectrum erythromycin and ampicillin was associated with prolongation of gestation compared to the absence of antibiotics (McGregor and French, Obstet. Gynecol. Clin. North Amer. 19:327-38, 1992). In contrast, a controlled study of prophylactic erythromycin therapy showed no decrease in the incidence of maternal or neonatal infectious morbidity; however, in patients "destined to have chorioamnionitis and oligohydramnios", pregnancy was significantly

prolonged (Mercer et al., Amer. J. Obstet. Gynecol. 166:794, 1992).

Only four trials evaluating the effect of antibiotics in treatment of women with preterm labor and intact membranes have been conducted. "Three of the four groups report an apparent prolongation of pregnancy with antibiotic therapy without impact on aggregate birth weight or perinatal mortality of the resultant infants" (Kirschbaum, Amer. J. Obstet. Gynecol. 168:1239, 1993). The results of these trials are difficult to interpret due to the use of different antibiotics, different clinical criteria for diagnosis of preterm labor and small numbers.

More promising strategies for reducing the incidence of preterm birth and lowering rates of neonatal morbidity and mortality may involve use of combination therapies, i.e., simultaneously using multiple, independent drugs. Kanayama (Nihon Sanka Fujinka Gakkai Zasshi. 44:110-15, 1992) reported a clinical study of women who showed signs of impending premature delivery. None of the patients had PROM, all were between the 24th and 35th weeks of pregnancy, and all had a "tocolysis index of 3-4", which was described as "imminent premature delivery of an intermediate degree." The tocolysis index is a relative index of delivery risk in which various risk factors for preterm delivery including status of amniotic membranes (rupture versus intact membranes), presence or absence of vaginal bleeding, estimation of cervical dilatation, and frequency of uterine contractions are semi-quantitatively assessed and scored, as indicated below.

	Tocolysis Index			
	0 pts	1 pt	2 pts	3 pts
Cervical Dilation	0 cm	1 cm	2 cm	3 cm
Vaginal Bleeding	none	---	spotting bleeding	
Ruptured Membranes	intact	---	---	rupture

Uterine Activity	none	irreg.	regular	---

The final tocolysis score represents the sum of each factor's "score" and hypothetically correlates to risk for preterm delivery as well as potential for successful tocolytic treatment. A tocolysis score of less than 3 indicates minimal risk for preterm delivery (and high probability of tocolytic success) while increasingly higher scores are associated with greater risk for preterm delivery (and lower probability of successful tocolytic intervention). While the tocolysis index is a modestly accurate method for assessing crude risk, it is neither reproducible between physicians (inter-observer error) nor a consistent predictor among individuals.

Terao and Kanayama evaluated the effect of four therapeutic strategies on preterm delivery rate as well as cervical expression of granulocyte elastase, a putative mediator of the labor process. The four therapeutic regimens evaluated included ritodrine infusion only (Group A), daily urinastatin vaginal suppositories (Group B), combination of ritodrine infusion and vaginal urinastatin suppositories (Group C), and combination of ritodrine infusion, vaginal urinastatin suppositories, and systemic antibiotic therapy (Group D). When patients were treated with urinastatin (groups B, C and D), the elastase in vaginal secretions decreased. The time required for the number of uterine contractions (UC) to decrease to less than 1 per 30 minutes was about an hour for groups A, C and D; whereas this same UC decrease took an average of about 6 hours in group B (urinastatin alone). When UC had been depressed for 4 days, therapy was discontinued. Approximately 60% of the patients in group A experienced a recurrence, whereas only 11-17% of the other groups experienced recurrent UC. In group A, 25% of women had premature deliveries, compared to no premature deliveries for

groups B and D (9 and 8 patients, respectively) and only 1/14 in group C.

However, in the above studies, no subjects with PROM or with tocolytic scores over 4 were tested. The authors suggested that "in more advanced cases of imminent premature delivery, further studies will be needed, since it is believed that localized therapies alone are insufficient." Moreover, there was no difference between group C (ritodrine and urinastatin) and group D (ritodrine, urinastatin and antibiotic).

Fuzishiro et al. reported that a patient with history of habitual abortion was observed to have a protruding amniotic sac at 20 weeks of gestation. She was treated with vaginal antibiotics and urinastatin, tocolytics and bed rest. Her pregnancy was maintained up to 36 weeks. Japan. J. Obstet. Gynecol. Neonatal. Hematol. 2:107-10, 1992.

What is needed is an effective method to prolong pregnancy, delay preterm delivery and reduce rates of neonatal morbidity and mortality in women with clinical signs of preterm labor.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods that are useful in delaying preterm delivery.

The role of calcium in muscle contraction is well established. Cytosolic free calcium combines with calmodulin and activates myosin light chain kinase, which results in phosphorylation of the myosin light chain. Phosphorylation of the myosin light chain in turn causes muscle contraction.

Calcium influx into uterine muscle is generally recognized as essential for uterine contraction. The increase of intracellular concentration of calcium is primarily due to calcium influx, as calcium storage in the endoplasmic reticulum is very low in smooth muscle cells. A fetus cannot emerge from the uterus through the birth canal

if the muscles of the uterus are relaxed, or uterine contraction is inhibited.

Thus, the present invention provides polypeptides derived from urinastatin (UTI) that have calcium channel blocking activity and that lack elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. The invention also provides polypeptides derived from urinastatin that have calcium channel blocking activity and have plasmin/cytokine/trypsin inhibiting activity and lack elastase inhibiting activity. The invention also includes methods of treating pregnant females to prevent imminent preterm delivery. The methods include administering a polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity.

Accordingly, one aspect of the invention is a polypeptide having the amino acid sequence RAF, said polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity.

Another aspect of the present invention is a polypeptide comprising the amino acid sequence NLPIVRGPCRAFIQL, said polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity.

In another aspect of the present invention, a polypeptide of about 15 amino acids comprising the sequence amino acid sequence RAF is provided.

Another aspect of the invention provides a polypeptide of about 15 amino acids in length comprising the amino acid sequence RAF, having a net positive charge of about two and a hydrophobic moment of about 0.56.

Another aspect of the invention is a polypeptide having the sequence NLPIVRGPCRAFIQL.

Another aspect of the present invention provides a polypeptide comprising the amino acid sequence RAF, said polypeptide having at most about 147 amino acids and having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. The polypeptide can also have at most about 75 amino acids or at most about 35 amino acids.

In another aspect of the invention, a polypeptide comprising Domain II of urinastatin is provided, said polypeptide having calcium channel blocking activity and plasmin/cytokine/trypsin inhibiting activity and lacking elastase inhibiting activity.

Another aspect of the present invention is a polynucleotide sequence encoding for the polypeptides, vectors that contain these polynucleotide sequences, and host cells that are transformed with these vectors.

Another aspect of the present invention provides a method for treating a pregnant female individual to delay imminent preterm delivery. The method comprises administering a polypeptide comprising the amino acid sequence RAF, said polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. This polypeptide can be administered alone or in conjunction with polypeptides derived from urinastatin having various activities, such as having plasmin/cytokine/trypsin inhibiting activity or having elastase inhibiting activity.

Another aspect of the invention is treating a female pregnant individual to delay imminent preterm delivery by administering a polypeptide having the amino acid sequence NLPIVRGPCRAFIQL. This polypeptide can be administered alone or in conjunction with polypeptides derived from urinastatin having various activities, such as having plasmin/cytokine/trypsin

inhibiting activity or having elastase inhibiting activity.

Another aspect of the invention is treating a female pregnant individual to delay imminent preterm delivery by administering a polypeptide comprising Domain II of urinastatin. This polypeptide can be administered alone or in conjunction with a polypeptide derived from urinastatin having elastase inhibiting activity.

Another aspect of the present invention is of diagnosing and treating a pregnant female individual at risk for impending preterm delivery. This method includes diagnosing by testing for a marker for impending preterm delivery. Females in whom imminent preterm delivery is indicated are administered a polypeptide comprising the amino acid sequence RAF, said polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. Alternatively, a polypeptide having the amino acid sequence NLPIVRGPCRAFIQL is administered. These polypeptides can be administered alone or in conjunction with polypeptides derived from urinastatin having various activities, such as having plasmin/cytokine/trypsin inhibiting activity or having elastase inhibiting activity. Alternatively, a polypeptide comprising Domain II is administered. This polypeptides can be administered alone or in conjunction with a polypeptide derived from urinastatin having elastase inhibiting activity.

In another aspect of the invention, a tocolytic agent and/or an antibiotic is administered in addition to the polypeptide(s).

In another aspect of this invention, there is provided the pharmaceutical composition comprising pharmacologically effective doses of the polypeptides and an antibiotic in a pharmaceutically acceptable excipient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid structure of UTI. Figure 1a depicts the various functional domains of UTI; Figure 1b shows the amino acid sequence of Domain II.

Figure 2 shows a tabular summary of the data for pregnant women undergoing treatment according to Example 2.

Figures 3a and 3b show effects of preincubated UTI on oxytocin or prostaglandin $F_2\alpha$ induced isometric uterine muscle contraction, respectively.

Figure 4 shows effects of preincubated UTI on LPS induced isometric uterine muscle contraction.

Figures 5a and 5b show the effect of UTI on the concentration of prostaglandin $F_2\alpha$ and E_2 in buffer, respectively, upon isometric contraction of uterine muscle stimulated by LPS.

Figures 6a and 6b show the effect of preincubation with fetal or adult urine, respectively, on endothelin-1 (ET-1) and prostaglandin $F_2\alpha$ induced muscle contraction.

Figure 7 shows the effect of UTI on $[Ca^{2+}]_i$ of uterine smooth muscle cells stimulated by LPS.

Figure 8 shows the effect of varying UTI concentrations on $[Ca^{2+}]_i$ in uterine smooth muscle cells stimulated by LPS.

Figure 9 shows the change in $[Ca^{2+}]_i$ in neutrophils by UTI treatment alone.

Figure 10 shows the effect of preincubation of neutrophils with UTI on the increase of $[Ca^{2+}]_i$ induced by LPS.

Figure 11 shows the effects of EGTA on the increase of $[Ca^{2+}]_i$ in the presence or absence of UTI.

Figure 12 shows the dose-dependent effect of UTI on the increase $[Ca^{2+}]_i$.

Figure 13 shows the effect of various UTI-derived polypeptides on $[Ca^{2+}]_i$.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides polypeptides having calcium channel blocking activity. The invention also provides methods for diagnosing and treating a pregnant female with impending premature delivery, with the treatment using the polypeptides.

Definitions

As used herein, the term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term "purified polypeptide" refers to a polypeptide or fragment thereof which is essentially free, i.e., contains less than about 50%, preferably less than about 70%, and even more preferably less than about 90%, of cellular components with which the polypeptide is naturally associated. Techniques for purifying polypeptides are known in the art, and examples of these techniques are discussed infra.

The term "derived from urinastatin" refers to a polypeptide sequence comprised of a sequence of at least about 5 amino acids corresponding to a region of the amino acid sequence of urinastatin. "Corresponding" means identical to or homologous to the amino acid sequence of urinastatin. The derived polypeptide may be generated in any manner, including but not limited to chemical synthesis or expression from a designated DNA sequence. In addition,

combinations of regions corresponding to that of the designated DNA or amino acid sequence may be modified in ways known in the art to be consistent with an intended use.

As used herein, "calcium channel blocking activity" is defined as inhibiting the influx of intracellular calcium ($[Ca^{2+}]_i$) after stimulation by known excitation agents. Certain substances are known to stimulate myometrial muscle contraction. These substances (excitation agents), such as such as oxytocin and prostaglandin F2α ($PGF_2\alpha$), thus provide a useful way to measure muscle contraction or inhibition of muscle contraction produced by a compound to be tested. Excitation agents include but are not limited to endothelin-1, prostaglandin $F_2\alpha$, oxytocin and lipopolysaccharide (LPS). Influx of $[Ca^{2+}]_i$ can be measured in cultured cells by digital imaging microscopy. Inhibition is established by at least about a 50% decrease in calcium influx in the presence of the polypeptide as compared to stimulation with one of the above-mentioned excitation agents in the absence of the polypeptide.

A "net positive charge" of a polypeptide means that the overall electrostatic charge for the entire molecule is positive rather than negative or neutral. The overall electrostatic charge of a polypeptide is obtained by summing the individual electrostatic charges of each of the component amino acids.

A "hydrophobic moment" means a measurement of the degree of hydrophobicity of a polypeptide. This measurement is usually expressed numerically and is compared to a scale, or index. This scale is also commonly known as the hydrophobicity index. Techniques for calculating this value are known in the art.

As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or

deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single- stranded DNA and RNA. Polynucleotides encompass RNA, cDNA, genomic DNA, synthetic forms, mixed polymers, both sense and antisense strands. It also includes known types of modifications, for example labels which are known in the art (e.g., Sambrook, et al.), methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl carbamate, etc.), those containing pendant moieties, such as for example, proteins (including for e.g., nuclease, toxins, antibodies, signal peptides, poly-$_L$ lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide. The polynucleotide may be chemically or biochemically modified or contain non-natural or derivatized nucleotide bases. The nucleotides may be complementary to the mRNA encoding the polypeptides. These complementary nucleotides include, but are not limited to, nucleotides capable of forming triple helices and antisense nucleotides. Recombinant polynucleotides comprising sequences otherwise not naturally occurring are also provided by this invention, as are alterations of wild type polypeptide sequences, including but not limited to, those due to deletion, insertion, substitution of one or more nucleotides or by fusion to other polynucleotide sequences.

A nucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to

produce the polypeptide or a fragment thereof. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

A polynucleotide sequence is "operably linked" when it is placed into a functional relationship with another DNA sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression. Generally, operably linked means that the sequences being linked are contiguous and, where necessary to join two polypeptide coding regions, contiguous and in reading frame. However, it is well known that certain genetic elements, such as enhancers, may be operably linked even at a distance, i.e., even if not contiguous.

The term "recombinant" polynucleotide or DNA refers to a polynucleotide which is made by the combination of two otherwise separated segments of sequence accomplished by the artificial manipulation of isolated segments of DNA by genetic engineering techniques or by chemical synthesis. In so doing one may join together DNA segments of desired functions to generate a desired combination of functions.

A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

"Treating" or "treatment" as used herein refers to prophylaxis and/or therapy.

"A tocolytic agent" is defined as a drug which is capable of suppressing uterine contractions. Examples of tocolytic agents include but are not limited to ritodrine, terbutaline, salbutamol (albuterol), nifedipine and indomethacin.

"An effective amount of a tocolytic agent" means that quantity sufficient to reduce the number of uterine contractions (UC) to about 1-2 in 30 minutes within not more than 3 hours.

As used herein, "an antibiotic" includes a compound capable of killing and/or stopping multiplication of microorganisms. The microorganisms which are of greatest concern in pregnant females are the natural flora of the vagina. The microorganisms which cause very serious infections in pregnant females include anaerobes. In general, unless an infection is present and the causative organism has been identified for treatment by a specific antibiotic, the present invention calls for the administration of a broad spectrum antibiotic. Examples of broad spectrum antibiotics include amoxicillin, ampicillin, erythromycin, azithromycin, and cephalosporins. Administration of more than one antibiotic and combinations of antibiotics are contemplated in the present invention. One such combination is UNASYN® ampicillin sodium/sulbactam sodium. Clindamycin is preferred as it is effective against anaerobes.

An effective amount of an antibiotic is that amount normally used to treat an infection. For example, the recommended dosage of UNASYN is 1.5 to 3.0 grams every six hours. The PHYSICIAN'S DESK REFERENCE, 47th ed., 1993, lists suitable antibiotics and recommended dosages.

"Preterm delivery" means childbirth before the fetus has reached about 36 completed (<37.0) weeks of gestation, as determined by patient history, ultrasound sizing, last menstrual period or other accepted methods.

"Impending or imminent preterm delivery" includes delivery of a fetus with a gestational age between 24 and 34 completed weeks which occurs within 7 to 10 days of the time of diagnosis of threatened preterm delivery or preterm labor. Impending preterm delivery needs to be identified, so that appropriate intervention can be undertaken to prevent premature delivery of a child whose immaturity contributes to mortality and morbidity. Impending preterm delivery is often associated with premature separation of the placenta and fetal membranes and/or premature uterine contractions.

A "marker for impending preterm delivery" is a substance that indicates a risk of imminent preterm delivery. Such risk is indicated by a correlation between the presence of or the elevation of the marker and preterm delivery. Examples of such markers include, but are not limited to, fetal fibronectin and elastase. The marker can also be a physical manifestation, such as tissue-specific changes in the cervix. Testing for a specific marker for impending preterm pregnancy includes all the usual methods for testing substances, including solid state chemistry, chromatography and specific antibody recognition techniques. Antibody recognition techniques include various sandwich antibody assays, using radio-, fluorescent- and enzyme-linked antibodies. In the ELISA test (enzyme-linked immunoassay), enzyme-linked

antibodies are subsequently exposed to chemicals on which enzymatic action causes formation of colored substances and/or substances detectable at a particular wavelength.

"Fetal fibronectin" is a unique fibronectin, found in amniotic fluid, placental tissue extracts and malignant cell lines. Fetal fibronectin is distinguished by an epitope termed the "oncofetal domain." This oncofetal domain is recognizable by a specific monoclonal antibody, which is used in a highly specific and sensitive test for fetal fibronectin.

"Significant amounts of fetal fibronectin" are levels which are above the concentrations commonly observed in the cervical or vaginal secretions of females with normal pregnancies between 24 and 34 completed weeks of pregnancy. In general, fetal fibronectin concentrations greater than about 50 ng/ml, as determined by a sensitive enzyme-linked immunoassay, are considered significantly elevated. Low levels of fetal fibronectin, i.e., concentrations of less than about 50 ng/ml, are present in the cervical and vaginal secretions of females with normal, uncomplicated pregnancies between 24 and 34 completed weeks. Although fetal fibronectin may be elevated before 24 weeks of pregnancy, this elevation is due to the normal development and growth of the placenta and does not necessarily reflect poor pregnancy outcome, e.g., preterm delivery, etc. Between 24 and 34 completed weeks, concentrations of fetal fibronectin greater than about 50 ng/ml in cervical or vaginal secretions are associated with preterm delivery.

"Analytical sensitivity" refers to the least amount of analyte that can be statistically distinguished from zero. In the present context, "clinical sensitivity" refers to the proportion of women who deliver prematurely with elevated fetal fibronectin concentrations. Preferably, the method

for identifying female subjects with impending preterm delivery has a clinical sensitivity of at least 80%.

"Analytical specificity" refers to the test's ability to report positive results due only to the presence of the desired analyte. Absence of "cross-reactivity" also refers to the same phenomena as "analytical specificity". In the present context, "clinical specificity" refers to the proportion of women with preterm deliveries in whom the fetal fibronectin concentration in cervical or vaginal secretions is greater than 50 ng/ml. Preferably, the method for identifying female subjects with impending preterm delivery has a clinical specificity of at least 80%.

"Individuals" are defined as humans and mammalian farm animals, sport animals and pets. Farm animals include, but are not limited to, cows, hogs and sheep. Sport animals include, but are not limited to, dogs and horses. The category pets includes, but is not limited to, cats and dogs.

As used herein, "an effective amount of a polypeptide" or "pharmacologically effective" means that the substance effects beneficial or desired clinical results. For purposes of this invention, a pharmacologically effective dose of a polypeptide means an amount which significantly delays impending preterm delivery. A pharmacologically effective dose can be administered either by a single administration or as a series of administrations. Doses are expressed in units, where one unit of polypeptide inhibits calcium influx by 50% as observed by the methods described herein. Preferably, 1000 to 10,000 units per dose are administered.

A "pharmaceutically acceptable excipient" is a relatively inert substance that facilitates administration of a pharmacologically effective substance. For example, an excipient can give form or consistency to the pharmaceutical composition, or act as a diluent. Suitable excipients include but are not

limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers. Examples of pharmaceutically acceptable excipients are described in Remington's Pharmaceutical Sciences (Alfonso R. Gennaro, ed., 18th edition, 1990).

GENERAL CONSIDERATIONS

The present invention provides polypeptides having calcium channel blocking activity. In one aspect of the invention, the polypeptides lack elastase inhibiting activity and lack plasmin/cytokine/trypsin inhibiting activity. In another aspect, the polypeptides have calcium channel blocking activity and plasmin/cytokine/trypsin inhibiting activity and lack elastase activity.

We have found that urinastatin (UTI) prevents uterine muscle contraction induced by oxytocin, endothelin-1 and prostaglandin by inhibition of calcium influx. More specifically, we have found that polypeptide fragments derived from UTI inhibit calcium influx. UTI has been sequenced and described in EP publication No. 0 100 985, published 1 August 1983, which is hereby incorporated by reference. The amino acid sequence and various functional domains of UTI are shown in Figure 1. The functional domains of UTI having elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity are disclosed in patent serial number PCT/US94/12751, attorney docket number 24612-20001.40, commonly owned, and incorporated herein by reference. UTI has two major domains. Domain II is a region of approximately 70 amino acids in length which exhibits calcium channel blocking activity and plasmin/cytokine/trypsin inhibiting activity and lacks elastase inhibiting activity. Domain II comprises amino acids of from about amino acid 80 to about amino acid 147 of the urinastatin sequence (Fig. 1a), and its amino acid sequence is shown in Figure 1b.

The elastase-inhibiting domain of UTI, designated 1B in Figure 1a, comprises the amino acids from about amino acid 36 to about amino acid 50 of the UTI amino acid sequence, and comprises the sequence MGMTSRYFYNGTSMA.

The plasmin/cytokine/trypsin-inhibiting domain of UTI, designated 2B in Figure 1a, comprises the amino acids from about amino acid 92 to about amino acid 106 of the UTI amino acid sequence, and comprises the sequence RAFIQLWAFDAVKGK.

In one aspect of the invention, the polypeptides comprise the amino acid sequence RAF. In another aspect, the polypeptide comprises the amino acid sequence NLPIVRGPCRAFIQL. In another aspect, the polypeptide is about 15 amino acids in length and comprises the amino acid sequence RAF.

The invention includes certain physical as well as functional characteristics of the polypeptides. Accordingly, another aspect of the invention is a polypeptide of about 15 amino acids and comprising the amino acid sequence RAF that also has a net positive charge of about two and a hydrophobic moment of about 0.56. The overall electrostatic charge of a polypeptide is obtained by summing the individual electrostatic charges of each of the component amino acids using techniques known by those of skill in the art.

Another aspect of the invention is a polypeptide having the amino acid sequence NLPIVRGPCRAFIQL. This polypeptide was found to completely inhibit the influx of intracellular calcium in LPS-stimulated neutrophils. An even smaller peptide, having the amino acid sequence RAF, was found to inhibit influx of intracellular calcium by about 70%. The results of these experiments are shown in Example 5.

This invention includes polypeptides derived from UTI comprising Domain II. Domain II was found to moderately inhibit the influx of intracellular calcium

in LPS-stimulated neutrophils, and has the amino acid sequence depicted in Figure 1b.

The invention encompasses polypeptides of varying lengths. The polypeptides of this invention can be as small as three amino acids in length. The polypeptides can also be larger, so long as they exhibit the requisite functional activity. For example, the polypeptide could comprise the amino acid sequence CRAF. The polypeptide could also comprise the amino acid sequence RAFI, CRAFI, CRAFIQ, or PCRAFIQ. These examples are not inclusive but serve to illustrate the various amino acid lengths encompassed by the invention. These examples include, but are not limited to, polypeptides of various lengths, wherein the polypeptide sequence comprises a portion of the amino acid sequence NLPIVRGPCRAFIQL, said portion comprising the sequence RAF.

Preferably, the polypeptide fragment will be at least approximately three amino acids in length and comprise the amino acid sequence RAF. The polypeptide fragment RAF has been shown to have calcium channel blocking activity. Even more preferably, the polypeptide will be approximately fifteen amino acids in length. As discussed above, polypeptides of varying lengths are included in the invention, as long as the polypeptides have calcium channel blocking activity and lack elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity.

In some embodiments of the invention, the polypeptides comprising the amino acid sequence RAF have at most about 147 amino acids, preferably at most about 75 amino acids, or even more preferably at most about 35 amino acids. The ideal polypeptide size is balanced between <u>in vivo</u> functionality and ease of commercial production.

The invention encompasses functionally equivalent variants of the polypeptides which do not significantly affect their properties and variants which retain the overall amino acid sequence but which

have enhanced or decreased activity. Thus, the polypeptides of the invention include substituted peptides which have calcium channel blocking activity and lack elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. Such polypeptides include polypeptides with conservative substitutions of amino acid residues, or one or a few deletions or additions of amino acids which do not change the functional activity. Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. These polypeptides also include glycosylated and nonglycosylated polypeptides, as well as polypeptides with other post-translational changes, such as glycosylation with different sugars, acetylation, etc.

The invention includes modifications to the polypeptide sequence (and thus the polynucleotide which encodes for the polynucleotide). For example, a polypeptide comprising the sequence abcdefghiRAFjkl can be produced, wherein positions a,b,c,d,e,f,g,h,i,j,k, and l are any amino acid, so long as the calcium channel blocking activity remains. Various amino acids can be inserted in the positions a-l. Preferably, the amino acid substitutions would be conservative, i.e., the substituted amino acid would possess similar chemical properties as that of the original amino acid. Such conservative substitutions are known in the art. However, a non-conservative amino acid substitution would be acceptable as long as the overall polypeptide has calcium channel blocking activity and lacks elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. The choice of amino acids will depend on conserving the overall function stated above.

In addition, the polypeptide need not contain the exact amino acid sequence as claimed. For example, another polypeptide encompassed by the invention would be the polypeptide of as delineated above, wherein the amino acid in position i is C or M. Another polypeptide would be abcdefgMRAFIkl, wherein positions a, b, c, d, e, f, g, k, and l, are any amino acid, and the peptide has calcium channel blocking activity and lacks elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. Any permutation of the above amino acid sequence would be encompassed by the invention, as long as the polypeptide displays the requisite functional properties.

Polypeptides thus obtained can be tested for their ability to inhibit the influx of intracellular calcium ($[Ca^{2+}]_i$) in stimulated cultured cells by digital imaging microscopy. One such assay uses cultured neutrophils and is described as follows. Cultured neutrophils which have been pre-loaded with Fura-2-AM, a fluorescing agent, are preincubated with the polypeptide to be tested. An excitation agent such as LPS is added, and the cells are irradiated by UV light at 340 nm and 380 nm periodically to provide Ca^{2+} independent fluorescence. The fluorescence ratios obtained from >10 cells are averaged at 30 second intervals. The sequential images are collected through a broad pass filter. A DC-stabilized Xenon lamp is fitted with a computer-associated excitation filter change. Video images are acquired by a silicon-intensified target camera. The output can be digitized by a color image analyzer, and the images integrated to improve the S/N ratio and calculated to the 340-380 nm ratio image on the image display. Image ratios are displayed as three-dimensional plots and temporal analysis lines and average ratio values. $[Ca^{2+}]_i$ is then calculated from the fluorescence ratio.

A polypeptide is characterized as having calcium channel blocking activity if addition of the polypeptide causes a decrease in the amount of calcium influx observed when the cells are treated by excitation agent alone. Preferably, the polypeptide will cause a 50% decrease. Even more preferably, the polypeptide will cause a 70% decrease.

The polypeptides of the invention also lack elastase inhibiting activity and/or plasmin/cytokine/trypsin inhibiting activity. Assays suitable for measuring these activities are known in the art. Typically, the activity is measured by providing a suitable substrate and measuring the activity in the presence or absence of the polypeptide(s) to be tested. Typically, the reaction is measured by detection of an end product. For example, the end product may be detected due to its chromogenic properties. Assays for determining activities at least one of the above functions are commercially available.

As an example, inhibition of plasmin activity is measured by using the chromogenic substrate S-2251.

The polypeptide(s) to be tested is preincubated with plasmin in a 96-well microtiter plate. Substrate S-2251 is added in a suitable buffer for effecting the reaction. The reaction is terminated by addition of 20% acetic acid, and the extent of the reaction is determined by measuring absorbance at 405 nm. Inhibition is determined by comparing this value to the value obtained when the reaction (under otherwise identical conditions) occurs in the absence of polypeptide.

A polypeptide is considered to exhibit inhibiting activity if the extent of the reaction in the presence of the polypeptide is about 50% of the extent of reaction in the absence of polypeptide. Preferably, the reaction in the presence of polypeptide is only about 30% of the extent of the

reaction in the absence of polypeptide. Even more preferably, the reaction in the presence of polypeptide is less than about 10% of the extent of the reaction in the absence of polypeptide.

The polypeptides of this invention can be made by procedures known in the art. The polypeptides can be produced by proteolytic or other degradation of the UTI molecule, by recombinant methods (i.e., single or fusion polypeptides) or by chemical synthesis. Preferably, the polypeptides are at least partially purified from other cellular constituents. Preferably, the proteins are at least 50% pure. More preferably, the proteins are 50-75% pure. More highly purified polypeptides may also be obtained and are encompassed by the present invention. For clinical use, the polypeptides are preferably highly purified, at least about eighty percent pure, and free of pyrogens and other contaminants. Methods of protein purification are known in the art and are not described in detail herein. Techniques for expressing the polypeptides alone or as fusion proteins are known in the art. Purification or isolation of the polypeptides expressed in host systems can be accomplished by any method known in the art.

Suitable methods of protein purification are known in the art and include, but are not limited to, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, HPLC and FPLC. Any purification scheme that does not result in substantial degradation of the polypeptide is suitable for use in the present invention.

The purified polypeptide can be obtained from urinastatin (UTI). UTI is a glycoprotein having an approximate molecular weight less than about 67kD in a purified form. At least a portion of urinastatin has been sequenced (EP publication No. 0 100 985, published 1 August 1983).

Urinastatin can be isolated from natural sources. Natural sources include, but are not limited

to, the urine of human men (as used by Mochida Pharmaceutical Co., Ltd., Tokyo, Japan). UTI may also be synthesized by either chemical or recombinant nucleic acid methods.

Urinastatin may be further processed to obtain the polypeptides of the invention. One such process is by proteolytic cleavage of the UTI molecule. Proteolytic cleavage is effected by digestion of UTI by various proteinases. Examples of proteinases include but are not limited to trypsin, plasmin, and thrombin. UTI can be incubated with one or more proteinases, or the digestions can be performed sequentially. The nature and extent of the proteolytic cleavage will depend upon the desired polypeptide length as well as the enzymes used. These techniques are well known in the art.

The polypeptides of this invention may also be produced by recombinant methods. Large quantities of the polypeptides may be prepared by transforming suitable prokaryotic or eukaryotic host cells with polypeptide(s)-encoding polynucleotides of the present invention in compatible vectors or other expression vehicles and culturing such transformed host cells under conditions suitable to attain expression of the polypeptide(s)-encoding polynucleotide sequence. The polypeptide(s) may then be recovered from the host cell and purified using standard techniques. The native expression may be suitable for small amounts of polypeptide and expression levels can be increased by upstream activation of expression. Methods of upstream activation are known and include insertion of activation sequences upstream of the polynucleotides encoding the polypeptide by homologous recombination. Techniques for nucleic acid manipulation useful for the practice of the present invention are described in a variety of references, including but not limited to, Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, eds. Sambrook et al. Cold Spring Harbor Laboratory Press (1989); and Current Protocols in

Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience: New York (1987) and periodic updates.

Another embodiment of this invention is a polynucleotide sequence encoding a polypeptide comprising the amino acid sequence RAF and having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. Alternatively, the polynucleotide sequence encodes a polypeptide comprising the amino acid sequence NLPIVRGPCRAFIQL. Another polypeptide encoded by the nucleotide sequence of this invention is a polypeptide having no more than about 147 amino acids and comprising the amino acid sequence RAF. Alternatively, the polypeptide can be only no more than 75 amino acids or 35 amino acids in length.

These polynucleotide sequences can be used in vectors for cloning and expression of recombinant polypeptides. In addition, it may be desirable to join one or more heterologous sequences to the polypeptide sequence. Such recombinant constructs can be useful for polypeptide production (i.e., secretion) or delivery (i.e., a more effective therapeutic modality). Methods for making such heterologous recombinant constructs are known in the art.

The invention includes modifications to the polynucleotide sequences such as deletions, substitutions and additions. Such changes are useful to facilitate cloning and modify gene expression.

The invention further embodies a variety of DNA vectors having cloned therein the polynucleotide sequence encoding the polypeptides described above. Suitable vectors include any known in the art including, but not limited to, those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors are known in the art and need not be described in detail herein.

Another embodiment of this invention are host cells transformed with vectors having polynucleotide sequences encoding the polypeptides. Both prokaryotic and eukaryotic host cells may be used. Prokaryotic hosts include *E. coli*. Among eukaryotic hosts are yeast and mammalian cells in culture systems. Host systems are known in the art and need not be described in detail herein.

In another embodiment, expression systems are provided for production of recombinant polypeptides. Expression systems are defined as polynucleotides which, when transformed into an appropriate host cell, can express a polypeptide(s). The polynucleotides possess a nucleotide sequence that is substantially similar to a natural polypeptide-encoding polynucleotide or a fragment thereof.

Expression systems prepared for introduction into a prokaryotic or eukaryotic host will typically comprise a replication system recognized by the host, including the intended DNA fragment encoding the desired polypeptide(s), and will preferably also include transcription and translational initiation regulatory sequences operably linked to the polypeptide encoding segment. Expression systems (expression vectors) may include, for example, an origin or replication or autonomously replicating sequence (ARS) and expression control sequence, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences, and mRNA stabilizing sequences. DNA encoding signal peptides may also be included where appropriate from secreted polypeptides of the same or related species, which allow the polypeptide of the invention to cross and/or lodge in cell membranes or be secreted from the cell.

The selection of an appropriate promoter and other necessary vector sequences will be selected so as to be functional in the host. Examples of workable

combinations of cell lines and expression vectors are described in Sambrook et al., 1989; Ausubel et al., 1987; and Metzger et al., Nature 334: 31, 1988. Many useful vectors for expression in bacteria, yeast, mammalian, insect, plant or other cells are well known in the art and may be obtained from such vendors as Stratagene, New England Biolabs, Promega Biotech, and others. In addition, the construct may be joined to an amplifiable gene (e.g., DHFR) so that multiple copies of the gene may be made. For appropriate enhancer and other expression control sequences see also Enhancers and Eukaryotic Gene Expression, Cold Spring Harbor Press, N.Y. (1983). While such expression vectors may replicate autonomously, they may less preferably replicate by being inserted into the genome of the host cell.

Expression and cloning vectors will likely contain a selectable marker, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the vector, although such a selectable gene may be carried on another polynucleotide sequence co-introduced into the host cell. Only those host cells into which the selectable gene has been introduced will survive and/or grow under selective conditions. Typical selection genes encode protein(s) that (a) confer resistance to antibiotics or other toxic substances, e.g. ampicillin, neomycin, methotrexate, etc.; (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media. The choice of the proper selectable gene will depend on the host cell, and appropriate genes for different hosts are known in the art.

The vectors containing the polynucleotides of interest can be introduced into the host cell by any of a number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile

bombardment; lipofection; infection (where the vector is an infectious agent, such as a retroviral genome). The choice of such means will often depend on the host cell.

The polypeptides of this invention may also be produced by chemical synthesis. Methods of chemical synthesis are known in the art and are commercially available. For example, the polypeptides could be produced by an automated polypeptide synthesizer employing the solid phase method.

Another embodiment of this invention provides a treatment method for impending preterm delivery. In some aspects of the invention, this method is preceded by a suitable test for preterm delivery (i.e., a method that tests for a marker for impending preterm delivery).

The treatment step comprises administration of a polypeptide comprising the amino acid sequence RAF and having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity. Alternatively, the treatment step comprises administration of a polypeptide comprising Domain II of UTI.

Fetal urine and amniotic fluid contain large amounts of urinastatin (Terao and Kanayama, Ibid.). This suggests that the origin of the urinastatin is the fetus. Since urinastatin occurs naturally in fetuses and pregnant women, it would also appear that polypeptides having the calcium blocking activity of UTI and various active polypeptide fragments of UTI would have the advantage of being a relatively safe drug for the fetus and pregnant woman.

It may be desirable to include in the treatment method administration of a tocolytic agent. The tocolytic agent may be any known tocolytic agents. The particular tocolytic agent is not critical to the invention. Beta-mimetic agents have been popular as tocolytic agents. Such beta-mimetic agents include,

but are not limited to, ritodrine, terbutaline, and albuterol (salbutamol). Other tocolytic agents are believed to suppress uterine contractility by inhibiting the release of intracellular calcium, including for example, magnesium sulfate and nifedipine calcium antagonist/calcium channel blocker. Anti-prostaglandins, such as indomethacin, have also been suggested for use as tocolytic agents but are effective for only a short time and have significant maternal and fetal toxicity.

The tocolytic agent generally is administered systemically. Although any systemic route of administration can be used, preferred routes include, but are not limited to, the intravenous and oral routes. The intravenous route may be preferred initially to develop high blood levels quickly; however, the oral route is often preferred, particularly if longer term maintenance therapy and drug administration are required.

The polypeptides may be administered individually or with other polypeptides derived from urinastatin that exhibit various activities. For example, a polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity can be administered alone or with a polypeptide having plasmin/cytokine/trypsin inhibiting activity. Alternatively, the polypeptide having calcium channel blocking activity can be administered with a polypeptide having plasmin/cytokine/trypsin inhibiting activity as well as with a polypeptide having elastase inhibiting activity. Another example is treatment with a polypeptide having calcium channel blocking activity and plasmin/cytokine/trypsin inhibiting activity alone or with a polypeptide having elastase inhibiting activity. Polypeptides that can be administered alone or in conjunction with other polypeptides include, but are not limited to, a polypeptide having calcium channel blocking activity

and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity, a polypeptide having elastase inhibiting activity, a polypeptide having plasmin/cytokine/trypsin inhibiting activity, and a polypeptide having calcium channel blocking activity and plasmin/cytokine/trypsin inhibiting activity (Domain II). A polypeptide derived from urinastatin having elastase inhibiting activity, comprising amino acids 36-50, or MGMTSRYFYNGTSMA, and a polypeptide derived from urinastatin having plasmin/trypsin/cytokine inhibiting activity, comprising amino acids 92-106 of urinastatin, or RAFIQLWAFDAVKGK, have been disclosed in patent serial number PCT/US94/12751, docket number 24612-20001.40, commonly owned. These examples are not inclusive but serve to illustrate the various treatment combinations encompassed by the invention. Obtaining smaller polypeptides that exhibit the requisite activity or activities is well within the skill of one in the art, and such methods have been described herein.

The polypeptides may be administered alone (as well as in conjunction with other polypeptides as discussed above) or with a tocolytic agent and/or an antibiotic. More preferred is their administration together. In a preferred embodiment the polypeptides are formulated in a vaginal suppository which also contains at least one antibiotic, preferably a cephalosporin. Another alternative is administration of one or both of the active polypeptides conjugated to an antibiotic. One such example is the pharmaceutical conjugated compound NLPIVRGPCRAFIQL-antibiotic. Even more preferably, the antibiotic of the conjugated compound is a cephalosporin. The conjugation of the polypeptides to the antibiotic can be performed by carboxyl and/or amide linkages using biochemical techniques well known in the art.

The polypeptides may be administered systemically or locally. More preferred is local

administration. Most preferred is intravaginal administration. Vaginal administration of the polypeptides can be performed in several other ways, including douche, suppository, foam and gel forms. In the form of a douche, an aqueous solution of polypeptides derived from urinastatin are directed against the cervix. Preferably, the polypeptides are administered in a solid or semi-solid form (foam, gel or suppository) which retains the polypeptides at the site of administration, preferably near the cervix.

The most preferred delivery form for the polypeptides is a vaginal suppository, which is a solid dosage form varying in weight and shape. After insertion, suppositories soften, melt or dissolve in the cavity fluids. Vaginal suppositories are usually globular or oviform and generally weigh about 5 grams. The usual suppository bases are cocoa butter, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights and fatty acid esters of polyethylene glycol. Use of cocoa butter (also known as theobroma oil or cocoa oil) is preferred. Cocoa butter melts quickly at body temperature.

Water-miscible suppository bases also may be used. Examples include polyethylene glycols or glycol-surfactant combinations. Polymers of ethylene glycol are available as polyethylene glycol polymers (Carbowax, polyglycols) of assorted molecular weights. Glycerinated gelatin is also often used as a vehicle for vaginal suppositories. Water-miscible suppository bases have the advantage of lack of dependence on a melting point approximating body temperature. Problems of handling, storage and shipping are simplified considerably.

Suppositories are prepared by well known methods including rolling (hand-shaping), molding (fusion) and cold compression.

Polypeptides of the invention are preferably administered as a single daily dose. However, they

also may be administered in two or more divided doses. Dosage ranges may be dependent upon the physiological responsiveness of the treated individual, and may be determined by the physician in charge of the treatment.

The antibiotic or combination of antibiotics contemplated in the present invention are those indicated by either diagnosis or suspected presence of microorganisms and include, for example, broad spectrum antibiotics and preferably antibiotics which are known to be effective against anaerobic bacteria. The particular antibiotic is not critical. Examples of broad spectrum antibiotics include amoxicillin, ampicillin, erythromycin, azithromycin, and cephalosporin. Clindamycin is preferred because it is effective against anaerobes. Use of more than one antibiotic, including combination antibiotics, is also contemplated in the present invention. One such combination is UNASYN® ampicillin sodium/sulbactam sodium (Roerig, Pfizer, Inc., New York, NY).

An effective amount of an antibiotic is that amount normally used to treat an infection. For example, the recommended dosage of UNASYN is 1.5 to 3.0 grams every six hours. The antibiotic can be administered systemically or locally. For systemic administration, oral administration is preferred; however, other methods, such as intravenous and intramuscular administration also can be used. For local administration, the antibiotic can be inserted vaginally, in the form of a cream, suppository or other suitable dosage form.

Various regimens for administering the antibiotic(s) can be utilized. For example, mezlocillin may be given intravenously for 48 hours and followed by oral ampicillin until the delivery. Other dual antibiotic regimens comprise intravenous ampicillin/oral erythromycin and ampicillin/gentamicin. A preferred three-antibiotic

regimen includes ampicillin, gentamicin and clindamycin.

The polypeptides of the invention can be administered for various periods of time. The length of treatment will vary depending on the condition of the individual patient, the length of pregnancy, and the repsonsiveness of the treated individual. In one regimen, the individual is administered an effective amount of a polypeptide(s) daily until 35 weeks. In another regimen the individual is also administered an effective amount of a tocolytic agent for less than about one week, and/or an effective amount of an antibiotic until the individual gives birth. Treatment can also include administering an effective amount of a tocolytic agent for less than about one week, and/or administering an effective amount of an antibiotic until the individual gives birth.

It is, of course, understood that the dosage, timing and administration requirements for the polypeptides alone or the polypeptides with a tocolytic agent and/or antibiotic may differ from subject to subject and are to be determined by the person responsible for treating the subject, without undue experimentation and using techniques known within the art.

In another aspect, the above-described treatment steps are preceded by the diagnosis of impending preterm delivery. Diagnosis comprises testing for females who are at risk for preterm delivery with a method which is sufficiently sensitive and specific for such a condition. Any suitable marker for preterm delivery can be used, so long as the test for the marker sufficiently distinguishes females at risk for preterm delivery from females who will not deliver early. Suitable markers for determination of impending preterm delivery include, but are not limited to, fetal fibronectin and elastase. A preferred test for impending preterm delivery has both specificity and sensitivity greater

than about 80%. A particularly preferred method is a test for fetal fibronectin. Most preferred is an ELISA test for fetal fibronectin. An ELISA test of this type may be performed as follows.

The mucosal sample to be tested is obtained from the cervix or the posterior fornix of the vagina on a swab. The swab is placed in buffer, in which the mucosal sample is diluted. Next, the concentration of fetal fibronectin is measured with an ELISA test (Fetal Fibronectin Immunoassay, Adeza Biomedical, Sunnyvale, CA). This assay utilizes a monoclonal antibody specific for the oncofetal antigen, followed by a goat anti-human plasma fibronectin IgG conjugated to alkaline phosphatase and a phenolphthalein monophosphate substrate. The absorbance of each standard and sample was determined at a wavelength of 550 nm with an automated microtiter-plate reader, and fetal-fibronectin concentrations were derived from the SoftMax software program (Molecular Devices, Menlo Park, CA). Values greater than about 50 ng/ml, determined during weeks 21-37 of pregnancy, are significant amounts and considered predictive of impending preterm delivery.

Another method for determining whether an individual is at imminent risk for preterm delivery is determining the concentration of elastase in the cervical and/or vaginal fluids. If the female individual has an abnormally high level of elastase, the female can be administered the polypeptide(s) of the invention with or without a tocolytic agent and/or an antibiotic.

The invention also encompasses pharmaceutical compositions of pharmacologically effective doses of the polypeptide(s) and an antibiotic in a pharmaceutically acceptable excipient. A pharmacologically effective dose is determined by testing various doses and treatment regimens of the composition. The pharmacologically effective dose

depends in part upon the manner of introduction to the individual as well as the indication to be treated.

This invention has been disclosed by direct description. The following examples are offered for illustration and should not be taken in any way as a limitation of the invention.

EXAMPLES

Example 1

Inhibition of calcium influx by UTI

Pyrogen-free urinastatin powder (Miraclid® brand) was obtained from Mochida Pharmaceutical Co., Tokyo, Japan. This powder was prepared from the fresh urine of healthy men, generally according to the method of Proksch and Routh (J. Clin. Lab. Med. 79:491, 1972). Purified UTI migrated as a single band in SDS-PAGE and in Sephadex gel chromatography.

Sample collection

Myometrial samples were collected from 20 full term normal pregnant women during elective Cesarean sections. The biopsy specimens were immediately immersed in chilled, oxygenated Krebs-Ringer bicarbonate buffer and transported to the laboratory.

Fetal urine was collected by urine bags from the first normal neonatal urine from babies who were delivered by Cesarean sections. After centrifugation of urine (1000 x g for 15 min.), the supernatant was collected and stored at -80°C. Adult urine was collected from 20 healthy volunteers, centrifuged and stored like fetal urine. Concentrations of UTI in fetal and adult urine were measured before use. Mean ±SD were 94.8 ± 4.7 U/ml and 10 ± 3.8 U/ml, respectively.

Isometric uterine contraction test

Myometrial samples were dissected into 3 x 7 mm strips in the direction of the muscle fibers under a stereo microscope. Only the middle strips from each patient were used. Silk ligatures were tied to each end of the muscle strip. One end was fixed to a

Plexiglas rod and mounted in a 30 ml organ chamber superfused by Krebs-Ringer bicarbonate buffer with 10 mM d-glucose, aerated by 95% O_2 + 5% CO_2. The free end of the strip was connected to a force transducer, and contractile activity was registered isometrically under a passive tension of 5 mN. The preparations were allowed to accommodate for 45 min before the start of the experiment. These strips were tested by oxytocin (1 μg/ml), prostaglandin $F_2\alpha$ (0.1 μg/ml), lipopolysaccharide (LPS) (10 μg/ml), endothelin-1 (0.1 μg/ml), UTI (100 U/ml=0.63 μM), fetal and adult urine.

During LPS stimulation for isometric contraction test, 1 ml of the buffer was collected every 20 min and stored at -80°C. Prostaglandin E_2 and $F_2\alpha$ in collected buffers were measured by radioimmunoassay as described. (Kanayama et al., Gynecol. Obstet. Invest. 28:123, 1989.)

Culture of smooth muscle cells

The biopsy specimens which were not used for isometric contraction test were cut to about 2 mm x 2 mm x 2 mm by scissors and each piece was placed on a thin cover glass and cultured in MEM medium containing 5% fetal calf serum. After the cells spread, attached and proliferated, tissue pieces were removed and cell cultures continued until approximately 80% of the cover slide was occupied by cells.

Digital imaging microscopy was carried out as described previously with some modifications (Yamamoto, Cell Struct. and Funct. 14:75, 1989) Briefly, myometrial cells on the cover glass were loaded by fura-2 AM for 30 min. The cultures were then put on the microscope stage previously warmed to 37°C by a thermostat heater controller. Before the experiment, cells were washed with phosphate-buffered saline, pH 7.3, and fetal calf serum-free MEM media was added (1h at 37°C). Cells in each disc were either directly stimulated by endothelin-1 or LPS or preincubated with UTI (500 nM) or fetal urine (100 μl) for 10 min and then stimulated by the same doses of

ET-1 or LPS. Final concentrations of 10 μg/ml endothelin-1 was added to the glass coverslip. The microscopic system consisted of a Nikon TMD-EFQ (Nikon, Japan) with a CF 100 x objective. The cells were excited by ultraviolet light at 340 nm and 380 nm excitations periodically to provide Ca^{2+} independent fluorescence. The fluorescence ratios obtained from >10 cells were averaged at 30 sec intervals. The sequential images were collected through a single broad pass filter (500 nm, band width 20 nm). A DC-stabilized Xenon lamp was fitted with a computer-associated excitation filter change. Video images were acquired by a silicon-intensified target camera (VIM-1, Hamamatsu Photonics K.K. Hamamatsu, Japan). The output was digitized by a color image analyzer ARGUS 100 (Hamamatsu Photoniotics K.K., Hamamatsu, Japan). The images were integrated to improve the S/N ratio and calculated to the 340-380 nm ratio image on the image display. Image ratios were displayed as three dimensional plots and temporal analysis lines and average ratio values $[Ca^{2+}]_i$ was calculated from the fluorescence ratio as described (Mohri and Hamaguchi, Cell Structure and Function, 16: 157, 1991).

Isometric uterine contraction test

Oxytocin, which stimulates myometrial contraction with increase in amplitude and frequency, did not induce contractions of smooth muscle in the presence of UTI (Fig. 3a). $PGF_2\alpha$, which stimulates myometrial contraction with increase in amplitude and frequency, did not induce contractions of smooth muscle in the presence of UTI (Fig. 3b). When UTI was added after stimulation of the muscle strip by prostaglandin $F_2\alpha$, muscle contraction was moderately inhibited (data not shown). LPS was found to stimulate uterine contraction synergetically with oxytocin and $PGF_1\alpha$. Similarly, uterine contraction stimulated with LPS was depressed in the presence of UTI (Fig. 4).

The concentrations of prostaglandin $F_2\alpha$ and E_2 were markedly increased in the buffer after LPS treatment. There was no significant change in their levels when UTI was preincubated (Fig. 5a, 5b). When the buffer in the organ bath was changed to fetal urine, the spontaneous uterine contraction was inhibited immediately. Incubation of the uterine muscles with fetal urine for 10-15 min rendered them non-responsive to ET-1 or $PGF_2\alpha$. After washing, uterine contraction spontaneously reappeared again in the presence of adult urine (Fig. 6a). Spontaneous uterine contraction depressed slightly and prostaglandin $F_2\alpha$ could induce uterine muscle contraction (Fig. 6b).

Cytosolic free Ca^{2+}

When UTI (>1 μM) was preincubated with uterine smooth muscle cells, the increase of intracellular Ca^{+2} concentration ($[Ca^{2+}]_i$) was completely inhibited in response to LPS stimulation (Fig. 7; color mapping data not shown). $[Ca^{2+}]_i$ of smooth muscle cells was increased up to 550 nM by LPS, but was unchanged in the presence of UTI (Fig. 7). Similar inhibition of the $[Ca^{2+}]_i$ increment was obtained by endothelin-1, prostaglandin $F_2\alpha$ and oxytocin in the presence of UTI (data not shown). The effect of increasing UTI doses on $[Ca^{2+}]_i$ is shown in Fig. 8. The concentration of 1000 nM UTI completely inhibited the increment of $[Ca^{2+}]_i$ by LPS (10 μg/ml).

Example 2

Combination therapies using UTI to delay imminent premature delivery

168 patients in their 24th to 35 week of pregnancy were diagnosed with imminent premature delivery. The diagnosis of imminent premature delivery was based on a tocolysis index of 2 or greater. Factors of cervical dilation, ruptured membranes, uterine activity and vaginal bleeding were considered for the tocolysis index. The tocolysis

index was determined by evaluating these factors and assigning points as shown in the following table. The index is the sum of these points.

	Tocolysis Index			
	0 pts	1 pt	2 pts	3 pts
Cervical Dilation	0 cm	1 cm	2 cm	3 cm
Vaginal Bleeding	none	---	spotting bleeding	
Ruptured Membranes	intact	---	---	rupture
Uterine Activity	none	irreg.	regular	---

A number of regimens were compared. Group 1 received ritodrine intravenous infusion at 50-300 μg per day. Groups 2 and 3 were given daily urinastatin suppositories containing 5000 units and 1000 units, respectively. Group 4 received IV ritodrine at 50-300 μg per day and one 1000 unit urinastatin suppository per day. Group 5 received IV ritodrine at 50-300 μg per day, one 1000 unit urinastatin suppository per day, and cephalosporin. The dose of urinastatin was expressed in units; one unit of urinastatin inhibits 2 μg of trypsin (3200 NFU/mg, Canada Packers, Toronto, Ontario, Canada) by 50% according to the method of Kassel (Methods Enzymol. 19:844, 1970). The patients were treated until they delivered.

A number of parameters were tracked and are reported in Figure 2. The tocolysis index immediately before the start of treatment varied from 2-8. Elastase levels obtained from specimen of endocervical secretions were determined at admission to the study. "Hours to Neg UA", or the time until the number of uterine contractions in 30 minutes decreased to one or less, was reported. "Recur UA", or the proportion of women experiencing recurrence of at least one uterine contraction every 30 minutes after therapy lasting 4 days or longer was discontinued also was observed. "EGA Toco" is the estimated gestational age at the beginning of therapy. "EGA Del" is the estimated

gestational age at delivery. The "interval" is the number of weeks between EGA Toco and EGA Del. "PTD<37 Wks" is the number of preterm births before 37 weeks. "PTD<34 Wks" is the number of preterm births before 34 weeks, or seriously premature infants.

All groups were comparable in maternal age, parity, estimated gestational age at start of study, tocolysis index and elastase level. However, group 5 had a relatively higher percentage of patients with tocolysis indices $\geq$6.

While treatment with ritodrine reduced uterine contractions more rapidly (1.4 hr compared to a study average of 3.1 hr) than other drug combinations, ritodrine alone had the highest rate of recurrence of uterine activity (over 30%) in spite of continuous administration of the drug. Finally, preterm deliveries of less than 34 and 37 wk accounted for approximately 21% and 42%, respectively, of all deliveries in women treated with ritodrine alone.

Women receiving only UTI suppositories (Group 3) experienced a slower expression of uterine activity than women receiving ritodrine, but in contrast, the recurrence rate of uterine activity was lower as was the proportion of deliveries occurring before 34 wk and 37 wk. As might be expected, combination of UTI and ritodrine rapidly suppressed uterine activity and diminished the recurrence of uterine activity. The proportion of deliveries occurring prior to 34 and 37 wk was not diminished compared to treatment with UTI alone. The combination of UTI, ritodrine and cephalosporin successfully suppressed uterine contractility, lowered the recurrence rate of uterine activity, and unexpectedly lowered the proportion of deliveries occurring before 34 and 37 wks.

Thus, the combination of ritodrine, urinastatin and cephalosporin was surprisingly more successful than the drugs alone or the combination of ritodrine and urinastatin.

Example 3

Comparison of therapies using polypeptide alone, polypeptide and tocolytic agent, and polypeptide and tocolytic agent and antibiotic

Patients are diagnosed as having risk of imminent preterm delivery by means of the fetal fibronectin immunoassay are treated with a combination of ritodrine (a tocolytic), a polypeptide derived from UTI having calcium channel blocking activity and cephalosporin antibiotic.

Mucous samples are obtained from women whose gestation period is less than about 34 weeks and who have signs and symptoms indicative of preterm delivery. The fetal fibronectin level is determined by a method which has both specificity and sensitivity greater than 80%. The mucous sample to be tested is obtained from the cervix or the posterior fornix of the vagina on a swab. The swab is placed in buffer, which dilutes the mucous sample. Next, the concentration of fetal fibronectin is measured with an ELISA test (Fetal Fibronectin Immunoassay, Adeza Biomedical). This assay utilizes a monoclonal antibody specific for the oncofetal antigen, followed by a goat anti-human plasma fibronectin IgG conjugated to alkaline phosphatase and a phenolphthalein monophosphate substrate. The absorbance of each standard and sample was determined at a wavelength of 550 nm with an automated microtiter-plate reader. Fetal-fibronectin concentrations were derived from the SoftMax software program (Molecular Devices). Patient with fetal fibronectin values greater than about 0.05 μg/ml, determined during weeks 21-37 of pregnancy, are considered to be at highest risk for impending preterm delivery (at risk of delivering within about seven days) and are entered into one of the following treatment groups.

Group A patients are given ritodrine drip infusion therapy; Group B patients are given daily polypeptide suppository therapy ; Group C patients are

given a combination of daily polypeptide suppository and ritodrine drip infusion therapy; and Group D patients are given a combination of ritodrine infusion, polypeptide suppository and oral cephalosporin therapy.

After treatment, the times to depress uterine contractions are monitored after four days of therapy. After four days of therapy, treatments are stopped but UC are monitored by a belt apparatus.

Example 4

Effect of various therapies on patients of varying risk profiles

Females whose fetal fibronectin values exceed 0.05 μg/ml during weeks 21-37 of pregnancy are randomly assigned to treatment groups to receive ritodrine alone, polypeptide alone or combination therapy of ritodrine, polypeptide and antibiotic as described in Example 3. For each treatment group, patients' fetal fibronectin values before the start of therapy are plotted against the rate of premature births to determine the effect of therapy on more or less risk-prone patients.

Example 5

Inhibition of calcium influx in neutrophils by polypeptides derived from UTI

UTI and neutrophil preparation

UTI was purified from human urine by the method of Proksch et al. (J. Lab. Clin. Med. 79:79) The purified UTI, which migrated as a single band on SDS-PAGE and on Sephadex gel chromatography, was provided by Mochida Pharmaceutical Co., Tokyo, Japan. Neutrophils were prepared as previously described (Yamamoto, ibid.). Briefly, 50 ml of fresh human blood obtained from four healthy donors was

anticoagulated with 5,000 units of heparin and immediately centrifuged through Ficoll-Hypaque. The cell pellet was resuspended and residual erythrocytes were lysed by using 0.16 M NH_4Cl containing 12 mM $NaHCO_3$ and 0.1 mM EDTA, pH 7.3. Neutrophils were washed with PBS. Neutrophils (5×10^6) in 1 ml of Hanks' buffer were put on a thin cover glass.

Effect of UTI peptides on increment of $[Ca^{2+}]_i$

Various UTI peptides were synthesized by the solid phase method and purified by C-4 HPLC using an acetonitrile gradient in 0.1% trifluoroacetic acid. Analytical C-4 HPLC monitored at 220 nm showed less than 2% impurity in all samples. The identity of the peptides was monitored by 40 MHz or 250 MHz ^1H NMR spectroscopy and mass spectroscopy for the peptides. The lyophilized synthetic polypeptides were dissolved in distilled water and pre-incubated with neutrophils for 10 min in a CO_2 incubator. Sequential concentrations of LPS (10 μg/ml) were added and $[Ca^{2+}]_i$ was measured same as described in Example 1.

Digital imaging microscopy was carried out as described in Example 1. After loading fura 2-AM for neutrophils, we added RPMI media containing 1 mM EGTA to the cells. UTI (1000 nM) was pre-incubated for 10 min at 37°C and LPS (10 μg/ml) was applied. The change of $[Ca^{2+}]_i$ was measured as above.

The change of $[Ca^{2+}]_i$ in neutrophils by UTI treatment only is shown in Fig. 9. After addition of UTI, $[Ca^{2+}]_i$ increased slightly up to 160 nM at 4 min and decreased gradually to basal level at 8 min. From 8 min, $[Ca^{2+}]_i$ of neutrophils was around 100 nM, and not changed. Preincubation of neutrophils with UTI (>1000 nM) completely inhibited increase of $[Ca^{2+}]_i$ induced by lipopolysaccharide (LPS) (Fig. 10). Under RPMI media containing EGTA, $[Ca^{2+}]_i$ was increased up to approximately 200 nM within 30 sec and decreased to 100 nM by LPS in neutrophils (Fig. 11). $[Ca^{2+}]_i$ of the cells was not changed by LPS in the presence of UTI. $[Ca^{2+}]_i$ influx was completely inhibited if cells were

preincubated for more than 10 minutes in UTI (data not shown). Figure 12 shows the dose-dependent inhibition of $[Ca^{2+}]_i$ influx by UTI.

The effects of synthetic peptides of UTI on $[Ca^{2+}]_i$ of neutrophils were investigated. The structure of UTI is shown in Fig. 1. Peptide 2A, in domain II, NLPIVRGPCRAFIQL (83-97), depressed Ca^{2+} influx completely like UTI (Fig. 13). Peptide 2B, RAFIQLWAFDAVKGK (92-106), inhibited increase in $[Ca^{2+}]_i$ moderately, but the other peptides tested did not inhibit increase in $[Ca^{2+}]_i$. The polypeptide RAF (92-94) resulted in 70% inhibition of the increment of cytosolic free Ca^{2+}.

Effect of serum on increment of $[Ca^{2+}]_i$

The change of $[Ca^{2+}]_i$ in HUVEC cells and neutrophils by LPS (10 μg/ml) was measured in the presence or absence of normal adult serum. Serum was incubated with UTI polyclonal antibody conjugated to sepharose beads for 2h at 30°C. After centrifugation, the supernatant was collected as sample. Serum treated with UTI antibody or untreated serum was pre-incubated with the cells at 30°C for 10 min. LPS was added to the wells. The change of $[Ca^{2+}]_i$ was measured as described above, but was assayed up to 10 min after stimulation.

Normal serum treated with ITI antibody that is cross-reactive with UTI (ITI, inter α trypsin inhibitor, is the precursor protein of UTI) increased in $[Ca^{2+}]_i$ of neutrophils by LPS and that without ITI antibody depressed increase of $[Ca^{2+}]_i$ (data not shown). The inhibition of increase of $[Ca^{2+}]_i$ in neutrophils was obtained in the presence of more than 10% serum concentration (data not shown).

This invention has been detailed both by example and by direct description. It should be apparent that one having ordinary skill in this art would be able to surmise equivalents to the invention as described in the claims which follow but which would be within the spirit of the description above.

Those equivalents are to be included within the scope of this invention.

CLAIMS

1. A purified polypeptide comprising the amino acid sequence RAF, said polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity.

2. A polypeptide according to claim 1 comprising the amino acid sequence NLPIVRGPCRAFIQL.

3. A polypeptide according to claim 1, wherein the polypeptide is about 15 amino acids in length and comprises the amino acid sequence RAF.

4. The polypeptide of claim 3, wherein the polypeptide has a net positive charge of about two and a hydrophobic moment of about 0.56.

5. A polypeptide having the amino acid sequence NLPIVRGPCRAFIQL.

6. A polypeptide according to claim 1, said polypeptide having at most about 147 amino acids.

7. The polypeptide of claim 6, wherein said polypeptide has at most about 75 amino acids.

8. The polypeptide of claim 7, wherein said polypeptide has at most about 35 amino acids.

9. A purified polypeptide derived from urinastatin, comprising Domain II.

10. The polypeptide of claim 9, wherein the amino acids comprise the sequence of Fig. 1b.

11. A polynucleotide sequence encoding a polypeptide selected from the group consisting of the

polypeptide encoded in claim 1, the polypeptide encoded in claim 2, the polypeptide encoded in claim 6, the polypeptide encoded in claim 7, the polypeptide encoded in claim 8, the polypeptide encoded in claim 9, and the polypeptide encoded in claim 10.

12. A vector comprising a polynucleotide sequence, wherein the polynucleotide sequence encodes for a polypeptide selected from the group consisting of the polypeptide from claim 1, the polypeptide from claim 2, the polypeptide from claim 6, the polypeptide from claim 7, the polypeptide from claim 8, the polypeptide encoded in claim 9, and the polypeptide encoded in claim 10.

13. A host cell transformed with the vector of claim 12.

14. A method for treating a pregnant female individual to delay imminent preterm delivery, said method comprising administering a polypeptide comprising the amino acid sequence RAF, said polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity.

15. The method of claim 14, further comprising administering a tocolytic agent.

16. A method according to claim 14, comprising administering the polypeptide of claim 5.

17. The method of claim 16, further comprising administering a tocolytic agent.

18. The method of claim 14, further comprising administering a polypeptide derived from urinastatin having plasmin/cytokine/trypsin inhibiting activity.

19. The method of claim 18, further comprising administering a tocolytic agent.

20. The method of claim 16, further comprising administering a polypeptide derived from urinastatin having plasmin/cytokine/trypsin inhibiting activity.

21. The method of claim 20, further comprising administering a tocolytic agent.

22. The method of claim 18, further comprising administering a polypeptide derived from urinastatin having elastase inhibiting activity.

23. The method of claim 22, further comprising administering a tocolytic agent.

24. The method of claim 20, further comprising administering a polypeptide derived from urinastatin having elastase inhibiting activity.

25. The method of claim 24, further comprising administering a tocolytic agent.

26. A method of claims 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25, further comprising administering an antibiotic.

27. A method for treating a pregnant female individual to delay imminent preterm delivery, said method comprising administering a polypeptide comprising Domain II.

28. The method according to claim 27, further comprising administering a tocolytic agent.

29. The method according to claim 27, further comprising administering a polypeptide derived

from urinastatin having elastase inhibiting activity.

30. The method according to claim 29, further comprising administering a tocolytic agent.

31. A method of claims 27, 28, 29, or 30, further comprising administering an antibiotic.

32. A method of diagnosing and treating a pregnant female individual at risk for impending preterm delivery, said method comprising the steps of (a) diagnosing imminent preterm delivery by testing for a marker for impending preterm delivery; and (b) if imminent pre-term delivery is indicated, administering to the individual a polypeptide comprising the amino acid sequence RAF, said polypeptide having calcium channel blocking activity and lacking elastase inhibiting activity and plasmin/cytokine/trypsin inhibiting activity.

33. The method of claim 32, further comprising administering a tocolytic agent.

34. A method according to claim 32, comprising administering the polypeptide of claim 5.

35. The method of claim 34, further comprising administering a tocolytic agent.

36. The method of claim 32, further comprising administering a polypeptide derived from urinastatin having plasmin/cytokine/trypsin inhibiting activity.

37. The method of claim 36, further comprising administering a tocolytic agent.

38. The method of claim 34, further comprising administering a polypeptide derived from urinastatin having plasmin/cytokine/trypsin inhibiting activity.

39. The method of claim 38, further comprising administering a tocolytic agent.

40. The method of claim 36, further comprising administering a polypeptide derived from urinastatin having elastase inhibiting activity.

41. The method of claim 40, further comprising administering a tocolytic agent.

42. The method of claim 38, further comprising administering a polypeptide derived from urinastatin having elastase inhibiting activity.

43. The method of claim 42, further comprising administering a tocolytic agent.

44. A method of claims 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, or 43, further comprising administering an antibiotic.

45. A method of diagnosing and treating a pregnant female individual at risk for impending preterm delivery, said method comprising the steps of (a) diagnosing imminent preterm delivery by testing for a marker for impending preterm delivery; and (b) if imminent pre-term delivery is indicated, administering to the individual a polypeptide comprising Domain II.

46. The method according to claim 45, further comprising administering a tocolytic agent.

47. The method according to claim 45, further comprising administering a polypeptide derived from urinastatin having elastase inhibiting activity.

48. The method according to claim 47, further comprising administering a tocolytic agent.

49. A method of claim 45, 46, 47, or 48, further comprising administering an antibiotic.

50. A pharmaceutical composition comprising pharmacologically effective doses of the polypeptide of claim 1 and of an antibiotic in a pharmaceutically acceptable excipient.

51. A pharmaceutical composition comprising pharmacologically effective doses of the polypeptide of claim 5 and of an antibiotic in a pharmaceutically acceptable excipient.

52. A pharmaceutical composition comprising pharmacologically effective doses of the polypeptide of claim 9 and of an antibiotic in a pharmaceutically acceptable excipient.

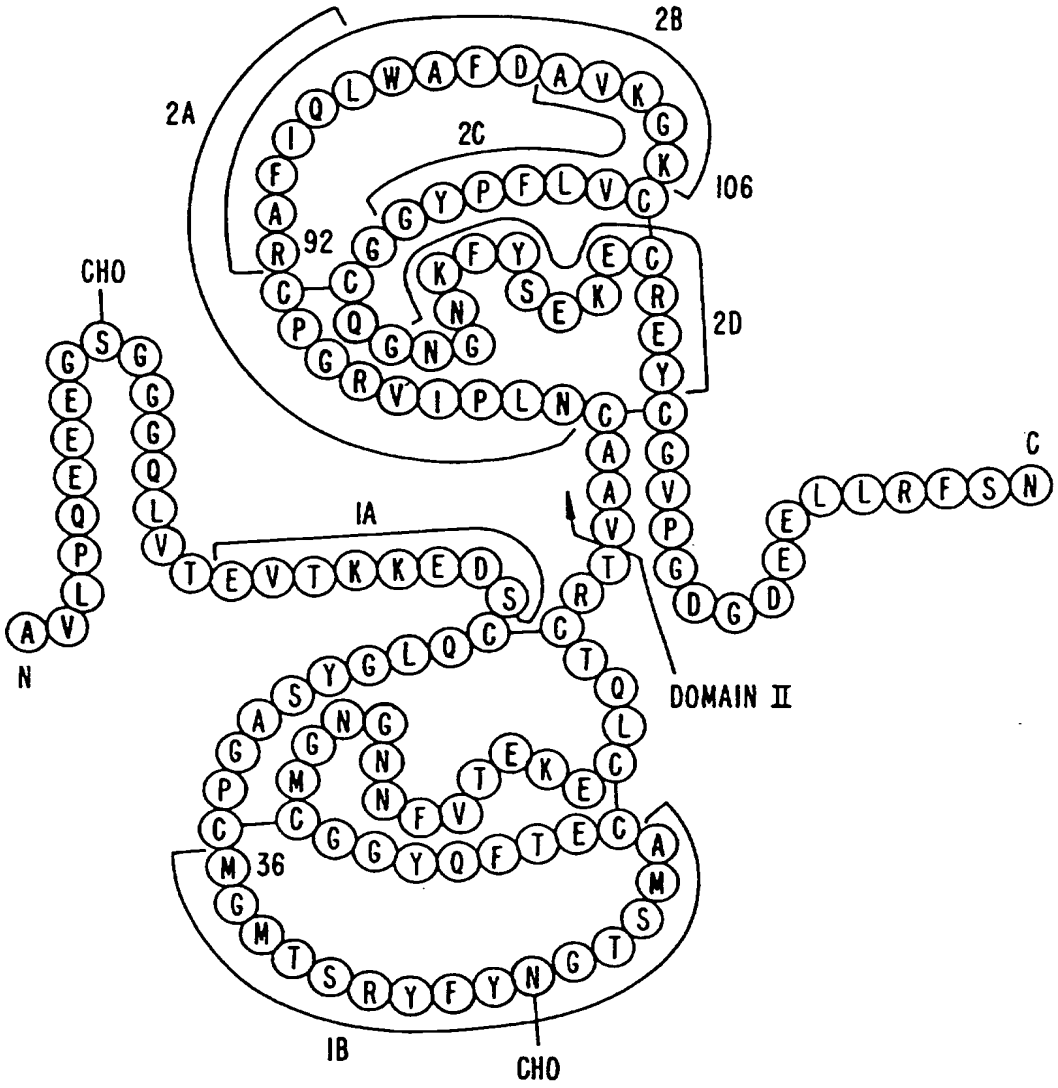

FIG. 1a.

VAACNLPIVRGPCRAFIQLWAFDAVKGKCVLFPYGGCQGNGNKFYSEKECREYCGVPGDGDEELLRFSN

FIG. 1b.

	GRP 1 n=66	GRP 2 n=14	GRP 3 n=33	GRP 2&3 n=47	GRP 4 n=25	GRP 5 n=30	ALL (n=168)
MATERNAL AGE (YR)	26.7 ± 5.0	26.7 ± 4.0	28.4 ± 6.1	27.9 ± 5.6	27.0 ± 5.0	27.7 ± 4.9	27.2 ± 5.2
PARITY n(%)							
0	27(40.9)	7(50.0)	11(33.3)	18(38.3)	11(44.0)	13(43.3)	69(41.1)
1	24(36.4)	3(21.4)	13(39.4)	16(39.4)	6(24.0)	12(40.0)	58(34.5)
2	10(15.2)	3(21.4)	5(15.2)	8(17.0)	5(20.0)	2(6.7)	26(15.5)
>2	5(7.6)	1(7.1)	3(9.1)	4(8.5)	3(12.0)	3(10.0)	15(8.9)
EGA TOCO (WEEKS)	29.5 ± 3.4	30.1 ± 3.2	28.4 ± 3.4	28.9 ± 3.4	29.2 ± 3.4	28.3 ± 3.0	29.1 ± 3.4
TOCO INDEX n(%)							
2	15(22.7)	4(28.6)	9(27.3)	13(27.7)	6(24.0)	8(26.7)	42(25.0)
3	18(27.3)	3(21.4)	11(33.3)	14(29.8)	8(32.0)	7(23.3)	47(28.0)
4	17(25.8)	4(28.6)	5(15.2)	9(19.1)	7(28.0)	8(26.7)	41(24.4)
5	8(12.1)	2(14.3)	4(12.1)	6(12.8)	2(8.0)	2(6.7)	18(10.7)
≥6	8(12.1)	1(7.1)	4(12.1)	5(10.6)	2(8.0)	5(16.7	20(11.9)
ELASTASE (mg/ml)	2.1 ± 1.5	2.4 ± 1.3	2.2 ± 1.6	2.2 ± 1.5	2.1 ± 1.3	2.0 ± 1.5	2.1 ± 1.5
HOURS TO NEG UA	1.4 ± 1.4	4.9 ± 3.3	5.7 ± 6.0	5.5 ± 5.3	1.8 ± 2.0	3.1 ± 3.4	3.1 ± 3.9
RECUR UA n(%)	21(31.8)	1(7.1)	4(12.1)	5(10.6)	4(16.0)	2(6.7)	32(19.0)
EGA DEL (WKS)	36.1 ± 3.4	38.2 ± 1.3	36.9 ± 3.1	37.3 ± 2.8	37.4 ± 2.7	37.3 ± 3.2	36.8 ± 3.2
INTERVAL (WKS)	6.5 ± 4.1	8.1 ± 3.8	8.5 ± 4.3	8.4 ± 4.2	8.2 ± 4.4	9.0 ± 3.5	7.7 ± 4.2
PTD < 37 WKS n(%)	28(42.4)	1(7.1)	7(21.2)	8(17.0)	5(20.0)	3(10.0)	44(26.2)
PTD < 34 WKS n(%)	14(21.2)	0(0.0)	5(15.1)	5(10.6)	2(8.0)	2(6.7)	23(13.7)

FIG. 2.

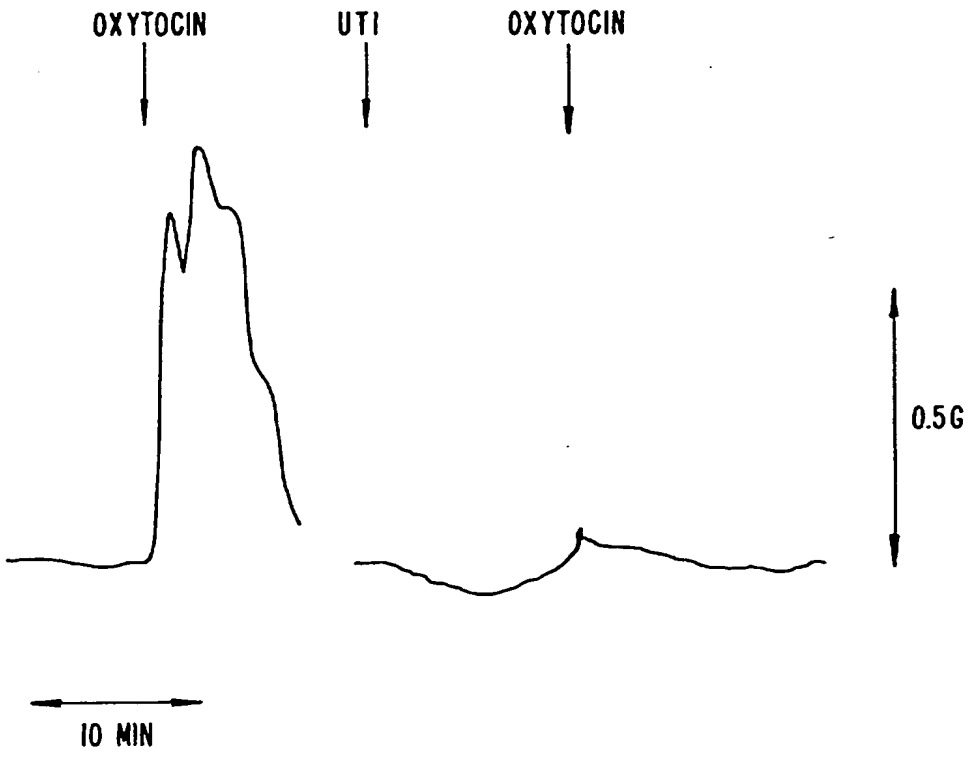

FIG. 3a.

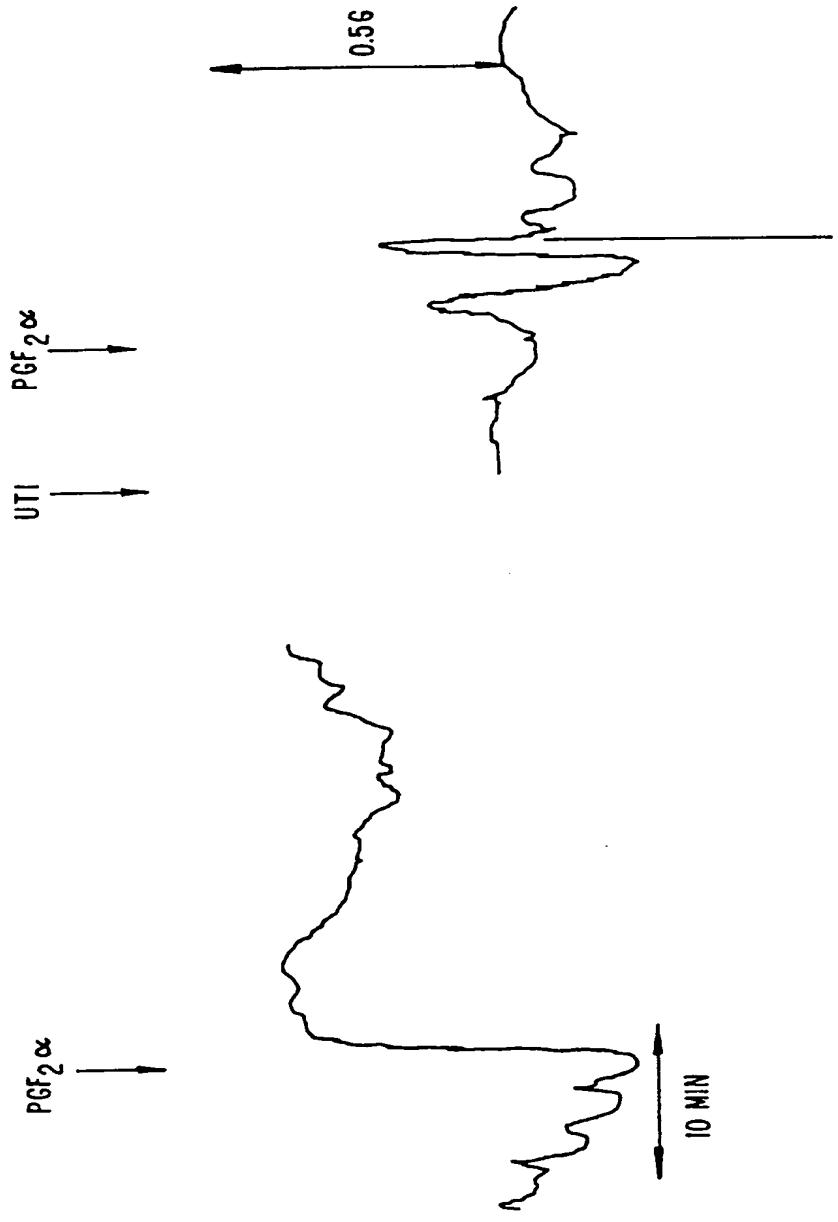

FIG. 3b.

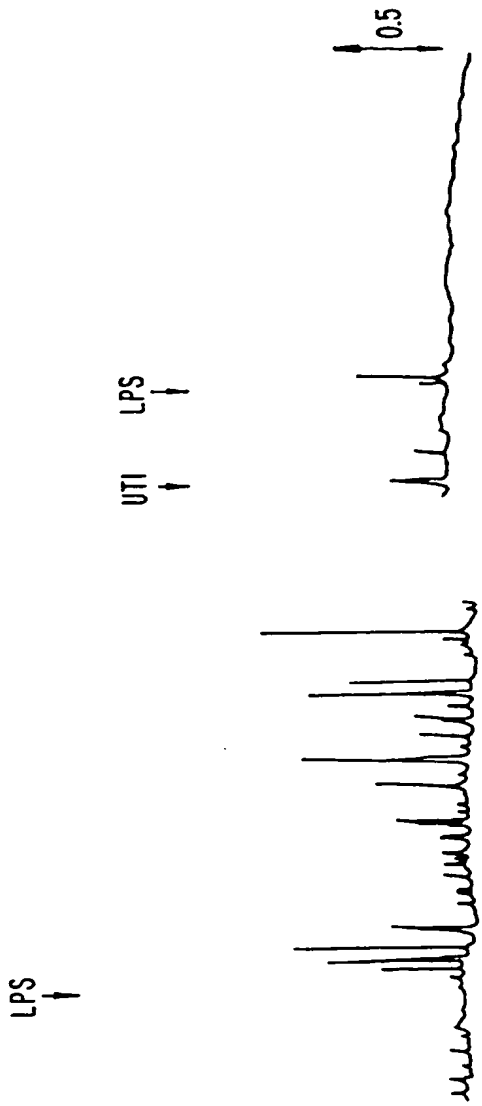

FIG. 4.

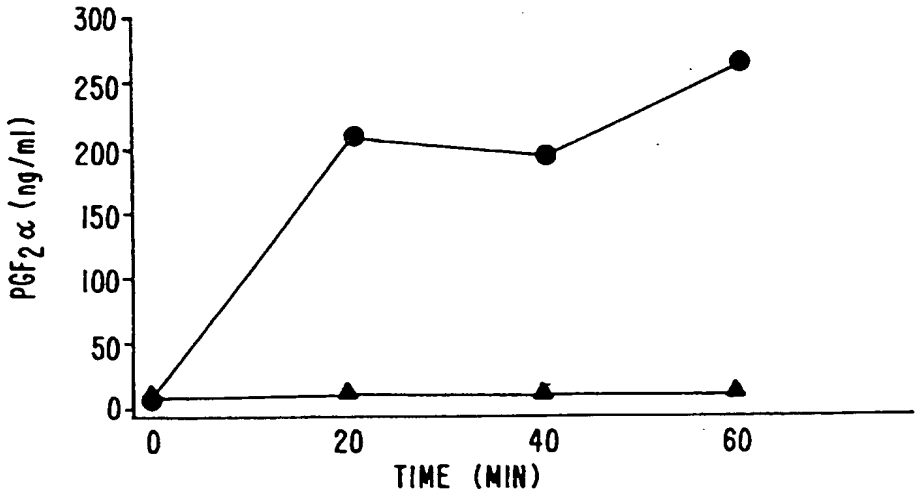
300
250
200
150
100
50
0
$PGF_2\alpha$ (ng/ml)
0
20
40
60
TIME (MIN)

FIG. 5a.

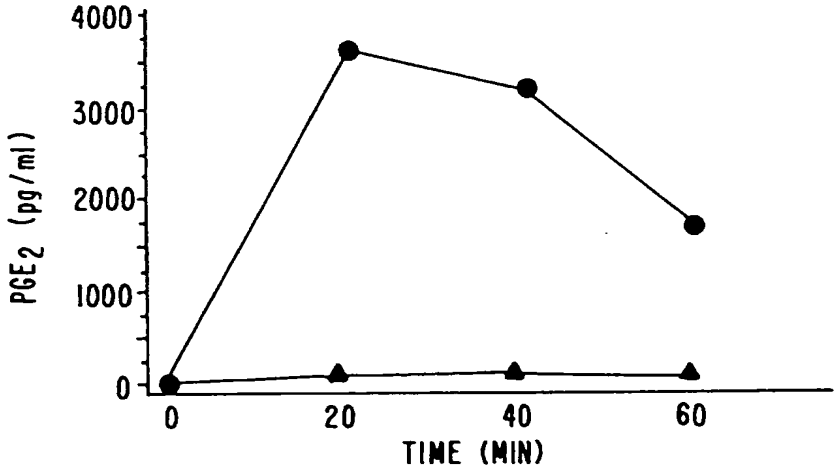
4000
3000
2000
1000
0
PGE_2 (pg/ml)
0
20
40
60
TIME (MIN)

FIG. 5b.

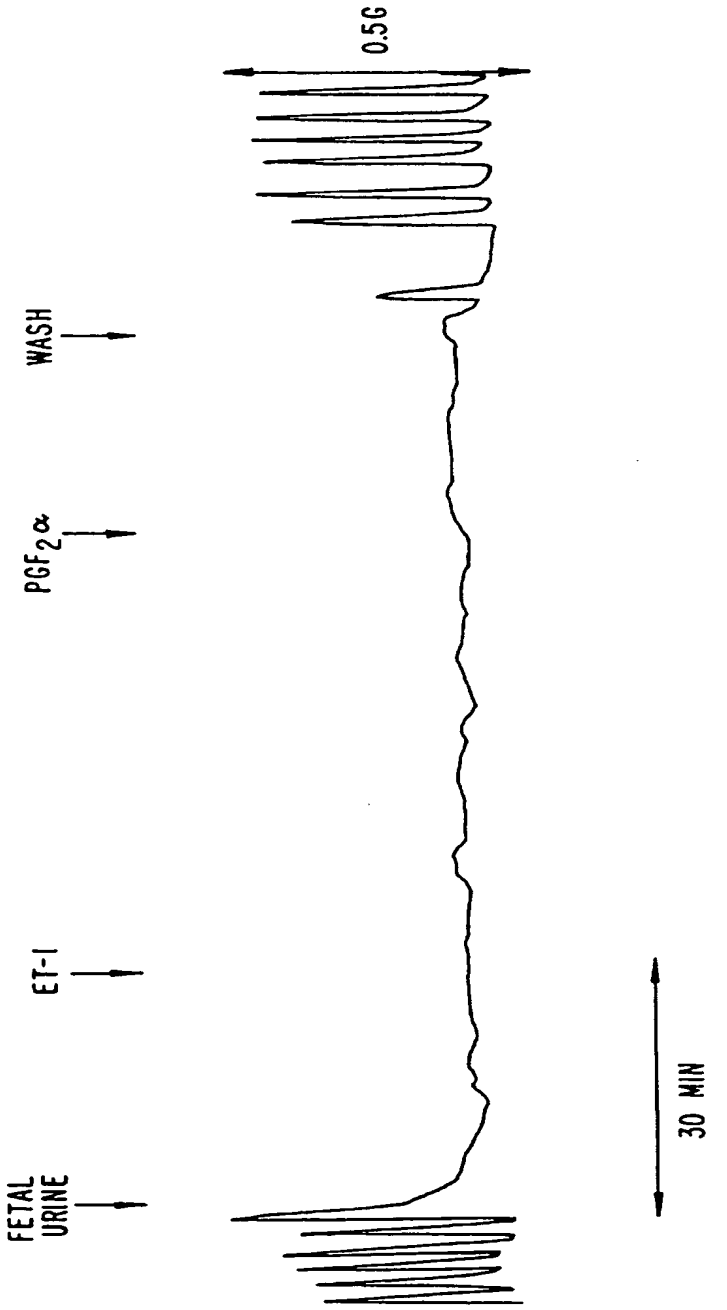

FIG. 6a.

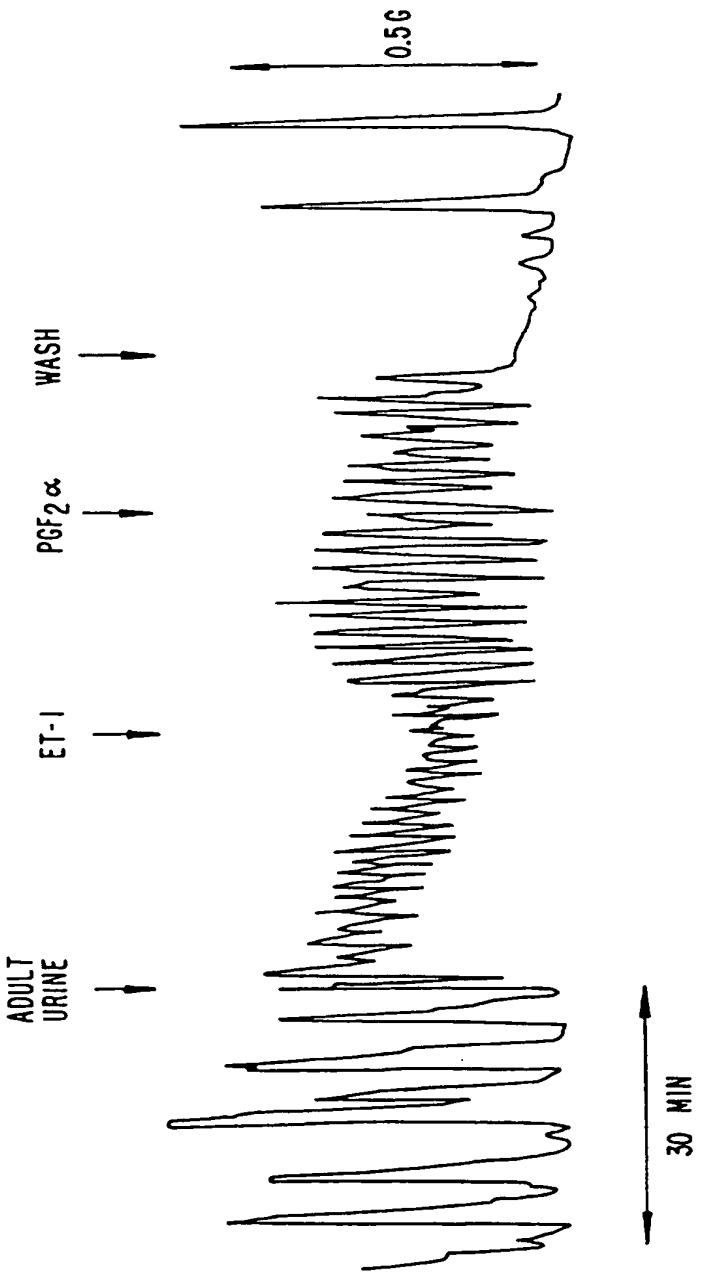

FIG. 6b.

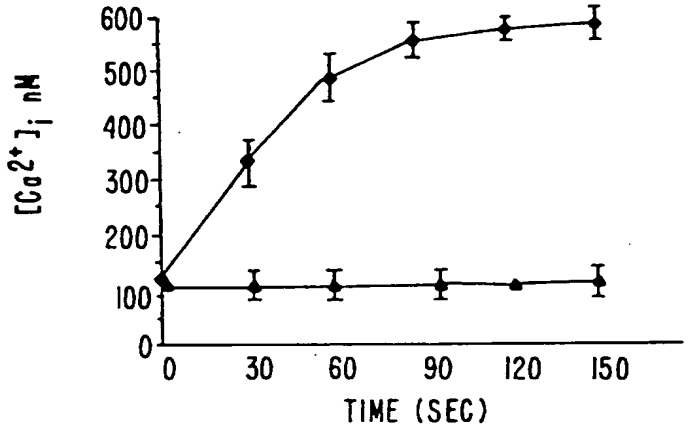
600
500
400
300
200
100
0
$[Ca^{2+}]_i$ nM
0
30
60
90
120
150
TIME (SEC)

FIG. 7.

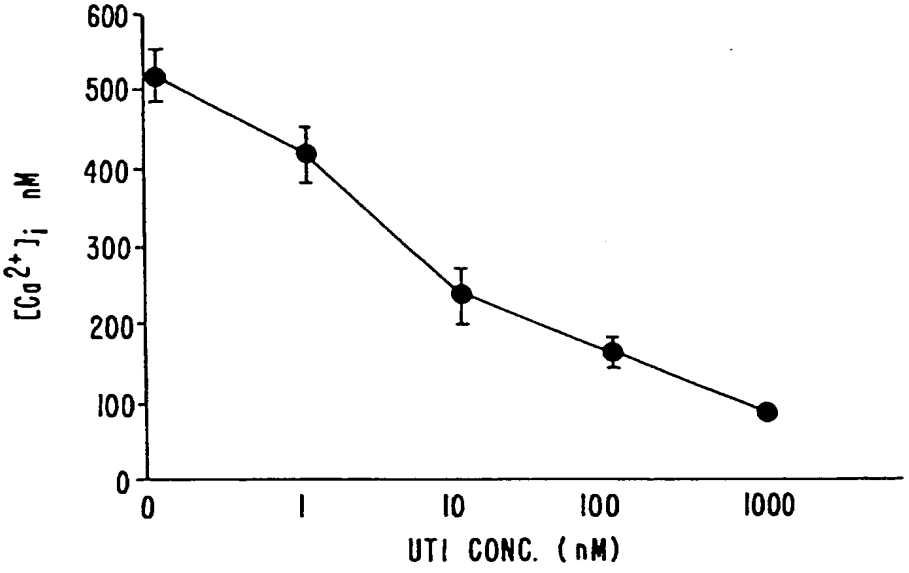
600
500
400
300
200
100
0
$[Ca^{2+}]_i$ nM
0
1
10
100
1000
UTI CONC. (nM)

FIG. 8.

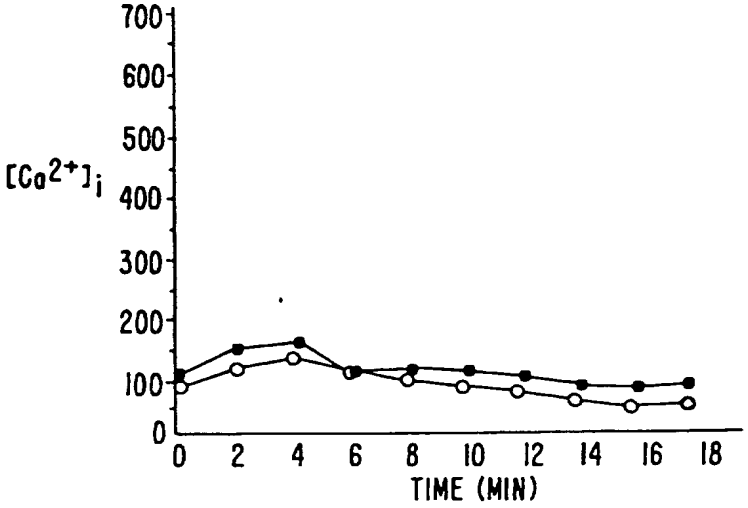

FIG. 9.

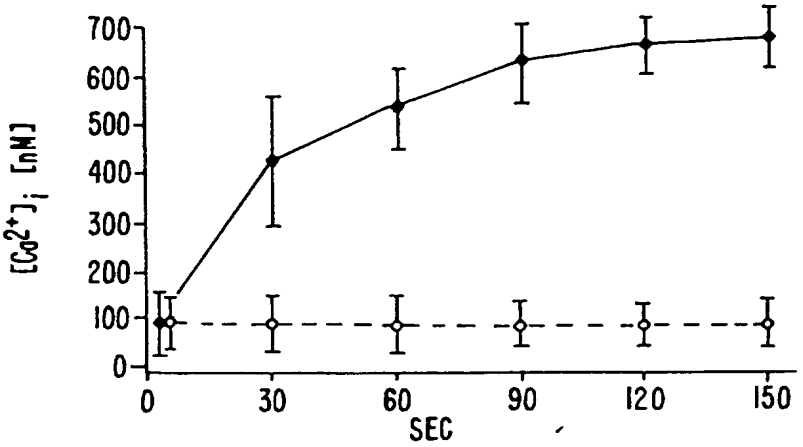

FIG. 10a.

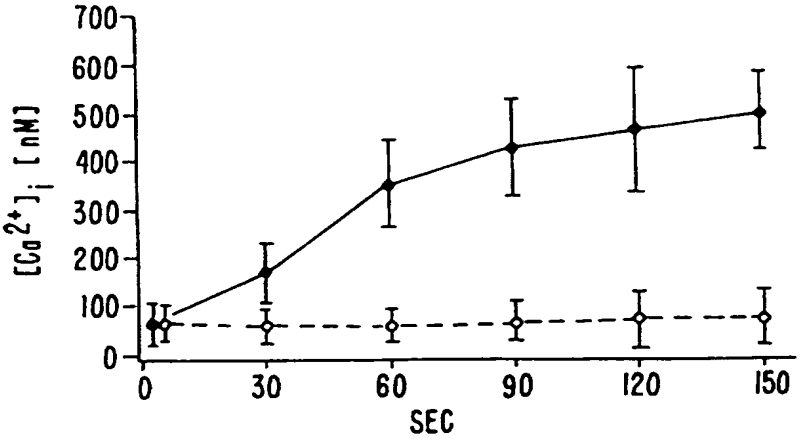

FIG. 10b.

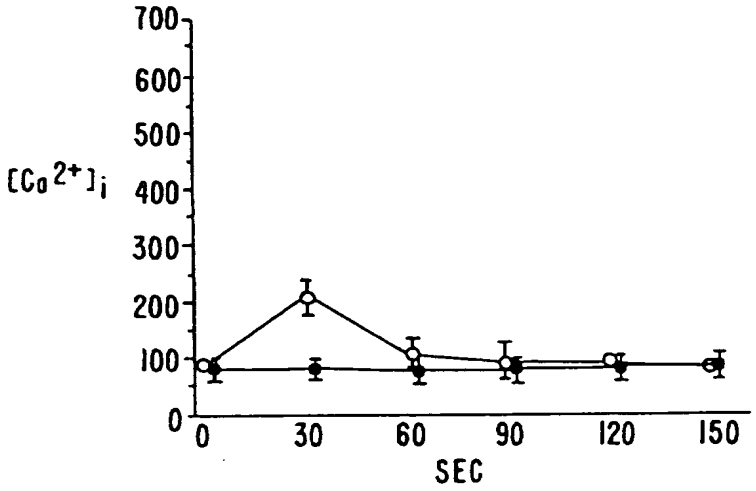
700
600
500
400
300
200
100
0
$[Ca^{2+}]_i$
0
30
60
90
120
150
SEC

FIG. 11.

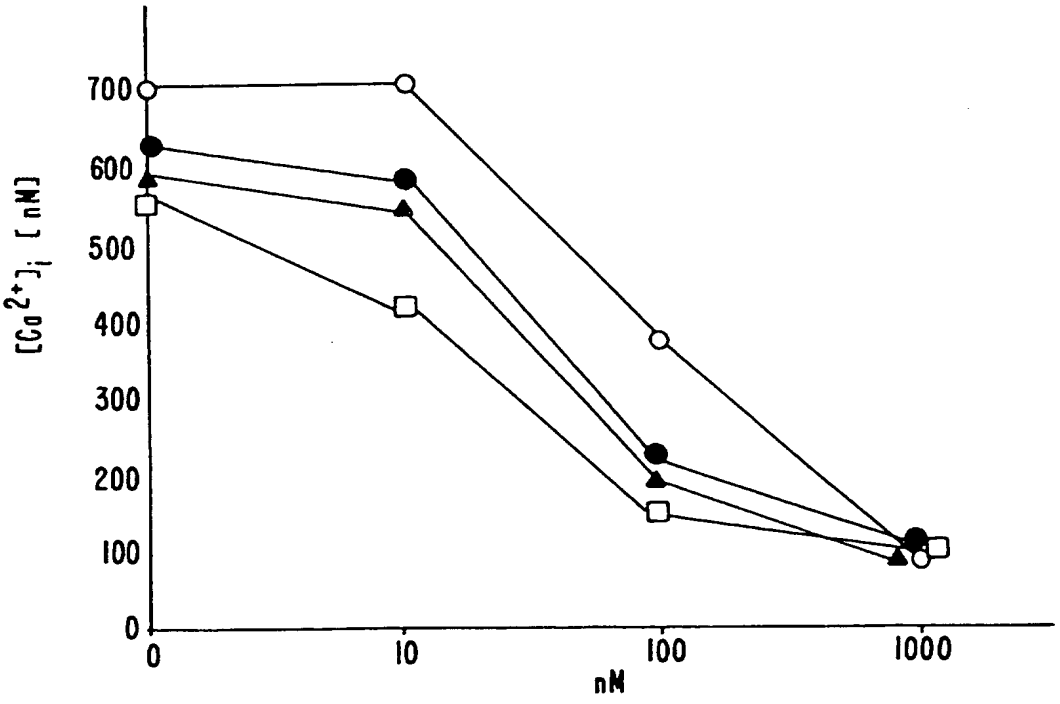
700
600
500
400
300
200
100
0
$[Ca^{2+}]_i$ [nM]
0
10
100
1000
nM

FIG. 12.

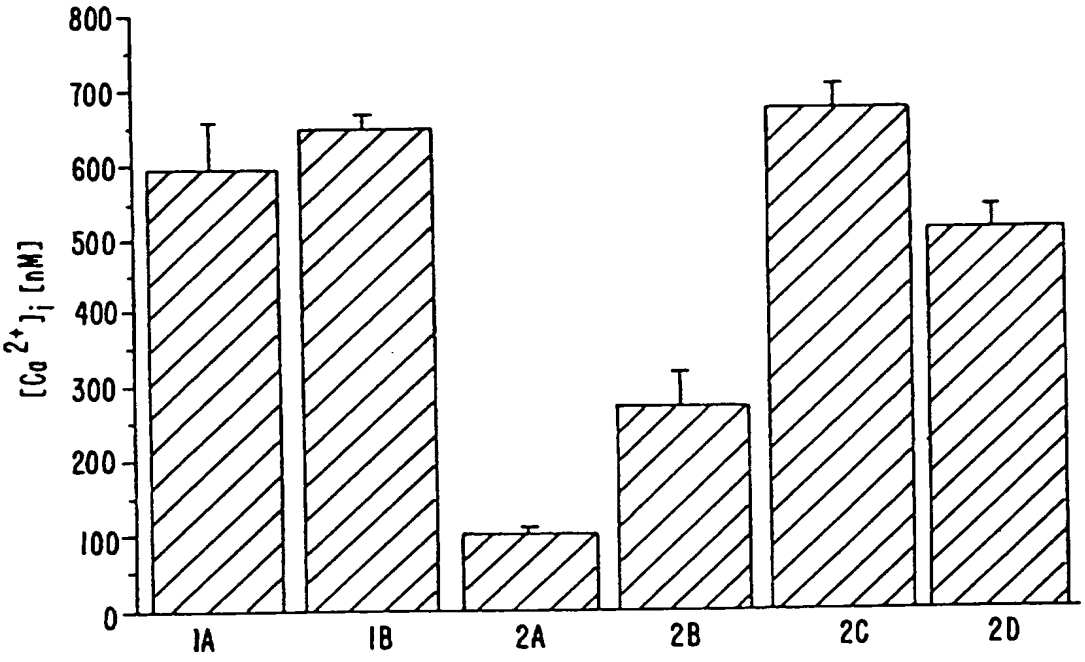

FIG. 13.

INTERNATIONAL SEARCH REPORT

Interr -nal Application No
PCT/US 96/02018

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/15 C12N5/10 C07K14/81 A61K38/57 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 12406 (ADEZA BIOMEDICAL ;TERAO TOSHIHIKO (JP); KANAYAMA NAOHIRO (JP); CAS) 11 May 1995 cited in the application see page 12, line 32 - page 13, line 5; claims; examples --- -/--	1,3,4, 6-8, 14-51

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1996	10.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Fuhr, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US 94376011, December 1994 N. KANAYAMA: "Intrauterine defensive mechanism of amniotic fluid and fetal membranes" XP002005590 see abstract & NIPPON SANKA FUJINKA GAKKAI ZASSHI. ACTA OBSTRETRICA ET GYNAECOLOGICA JAPONICA, vol. 46, no. 8, August 1994, pages 673-765, ---	1,2,9, 10, 14-50,52
X	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US 92300191, September 1992 N. KANAYAMA ET AL.: "The effect of granulocyte elastase inhibitor (urinastatin) vaginal suppository on patients with imminent premature delivery" XP002005699 see abstract & NIPPON SANKA FUJINKA GAKKAI ZASSHI. ACTA OBSTETRICA ET GYNAECOLOGICA JAPONICA, vol. 44, no. 4, April 1992, pages 477-482, ---	1,2,9, 10, 14-50,52
X	EP,A,0 486 001 (MOCHIDA PHARM CO LTD) 20 May 1992 see claims; examples ---	1,2,4,6, 7,9-13, 50,52
X	EP,A,0 624 644 (MOCHIDA PHARM CO LTD) 17 November 1994 see formula 23 on page 15 see claims; examples ---	1,2,4,6, 7,9-13, 50,52
X	EP,A,0 543 240 (MOCHIDA PHARM CO LTD) 26 May 1993 see claims; examples ---	1,2,4,6, 7,9-13, 50,52
X	PATENT ABSTRACTS OF JAPAN vol. 18, no. 114 (C-1171), 24 February 1994 & JP,A,05 308988 (MOCHIDA PHARMACEUT CO LTD), 22 November 1993, see abstract --- -/--	1,2,4,6, 7,9-13, 50,52

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 96/02018

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 401 508 (BAYER AG) 12 December 1990 see claims; examples ---	1,2,4,6, 7,9-13, 50,52
P,X	EUROPEAN JOURNAL OF OBSTETRICS & GYNECOLOGY AND REPRODUCTIVE BIOLOGY 60 (2). 1995. 181-186. ISSN: 0301-2115, June 1995, XP000572784 KANAYAMA N ET AL: "Urinary trypsin inhibitor suppresses premature cervical ripening." see page 185, right-hand column, paragraph 2 - page 186, left-hand column, paragraph 1 -----	1-10,14, 50-52

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US96/02018

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14-49
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: As far as claims 14-49 are directed to a method of treatment of and/or diagnostic method practised on the human and/or animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inv ional Application No
PCT/US 96/02018

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9512406	11-05-95	AU-B-	1050495	23-05-95
EP-A-0486001	20-05-92	AU-B-	641568	23-09-93
		AU-B-	8783991	11-06-92
		CA-A-	2055425	14-05-92
		JP-A-	5084083	06-04-93
		US-A-	5409895	25-04-95
EP-A-0624644	17-11-94	JP-A-	6315386	15-11-94
		AU-B-	6181494	03-11-94
		CA-A-	2122556	02-11-94
EP-A-0543240	26-05-93	AU-B-	661515	27-07-95
		AU-B-	2820992	13-05-93
		CA-A-	2082226	09-05-93
		JP-A-	6025289	01-02-94
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		CA-A-	2016627	13-11-90
		DE-D-	59007737	05-01-95
		ES-T-	2066033	01-03-95
		JP-A-	3255099	13-11-91
		US-A-	5407915	18-04-95